STUDIES WITH TEMPERATURE-SENSITIVE MUTANTS OF SEMLIKI FOREST VIRUS

BY

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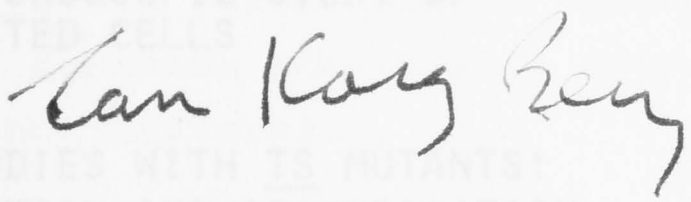
STATEMENT

Studies with temperature-sensitive mutants of Semliki Forest virus were started in 1965 by Dr J.F. Sambrook and continued by me in 1966. The 5-Fluorouracil and hydroxylamine induced mutants used in the present studies were isolated by Dr J.F. Sambrook and Miss N.A. Hodge; the rest of the mutants were isolated by me.

Some of the experiments reported in Chapter 4 were carried out in collaboration with Dr A.J.D. Bellett. Polyacrylamide gel electrophoresis of SFV nucleocapsids reported in Chapter 5 was performed in collaboration with Dr C.J. Burrell.

All the other work reported in this thesis was carried out by me.



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SUMMARY

The studies of temperature-sensitive mutants of Semliki Forest virus reported in this thesis are contained in seven chapters. Three other chapters are devoted to a general introduction, the materials and methods used and a general discussion and conclusions.

Semliki Forest virus was chosen as a model for the study of the replication and genetics of the group A arboviruses. These are small spherical riboviruses consisting of an internal core or nucleocapsid covered with an envelope and their morphology, biochemical properties, replication and genetics are reviewed in Chapter 1. The study of Semliki Forest virus was carried out with temperature-sensitive or ts mutants which were derived from the wild-type virus after treatment with mutagens. These mutants multiplied at a low (permissive) temperature but not at a high (restrictive) temperature and have been shown to be invaluable for the study of the physiology and genetics of a number of animal viruses.

Chapter 2 is on 'Materials and Methods'.

The isolation and growth characteristics of Semliki Forest virus ts mutants are described in Chapter 3. A system for studying viral growth in the absence of host cell's macromolecular biosynthesis is reported.

The replication of Semliki Forest virus involves first the synthesis of viral RNA and then the synthesis of viral structural proteins. The ability of the mutants to synthesise RNA at the restrictive temperature is reported in Chapter 4. One group of mutants failed to synthesise RNA (RNA^- mutants), others ($RNA^{\pm}$ and RNA^+ mutants) made from five to 90 per cent of the wild-type virus

yield of RNA at the restrictive temperature. Wild-type virus made four species of RNA in infected cells; one is incorporated into virions, two are intermediates in viral RNA replication and another RNA of unknown function. $RNA^{\pm}$ and RNA^{+} mutants made all these RNAs but in proportions different from those of wild-type virus. RNA^{-} mutants were used to test if inhibition of cellular RNA synthesis could occur in the absence of viral RNA synthesis.

Two major proteins are found in the virions of Semliki Forest virus, the nucleocapsid protein and the membrane protein in the envelope. $RNA^{\pm}$ and RNA^{+} mutants were found to be defective in the production of these structural proteins or in their maturation to form virions at the restrictive temperature (Chapter 5). Viral antigens produced in cells infected with mutants were investigated by staining the cells with specific fluorescent antibody.

The temperature-sensitive defects of RNA^{-} and RNA^{+} mutants were expressed at different times of the viral growth cycle (Chapter 6). RNA^{-} mutants were defective in a function expressed early (within first four hours) in the growth cycle, presumably the synthesis of viral RNA polymerase. RNA^{+} mutants were defective in functions expressed late (after four hours) in the growth cycle; these functions have been identified in the previous chapter.

Chapter 7 reports investigations on the defects in the virions of the mutants. Two properties of the virions were tested, stability of infectivity to heat inactivation and hemagglutination (HA) activity. Most

RNA^- mutants were heat stable (at 50.5^o) whereas most RNA^+ mutants were heat labile. A few RNA^+ mutants produced, at the permissive temperature, virions which had reduced HA activity.

Cells infected with three mutants at the restrictive temperature were studied in the electron microscope (Chapter 8) to correlate their morphological and physiological defects; a close correlation was found.

Chapter 9 describes complementation and recombination tests with the mutants. It was hoped that these tests would enable mutants to be placed in different functional groups or cistrons (by complementation tests), and the genes mapped (by recombination tests). However, complementation was inefficient and recombination was not easy to demonstrate.

Chapter 10 collates the experimental results which are discussed with particular reference to the mechanism of replication of Semliki Forest virus.

CHAPTER 1

GENERAL INTRODUCTION

Viruses provide extremely useful systems for the study of gene functions and structure. Deoxyriboviruses, like cells, replicate with the DNA functioning as a template for the synthesis of new components. The replication of riboviruses in cells involves a novel system with viral RNA functioning as a template for its own biosynthesis. This novel RNA replication mechanism has recently been demonstrated for a number of small animal riboviruses.[54]

The group A arboviruses offer a promising system for the study of the replication of riboviruses. Their ability to grow in a large number of tissue culture systems and over a wide range of temperature, should facilitate the isolation of 'conditional lethal mutants' which have been shown to be invaluable in the study of phage genetics and physiology.[25] The small genome of arboviruses of molecular weight of about $2x10^6$ daltons is not expected to code for more than 12-15 polypeptides so that a complete characterization of gene products and mapping of the genes are feasible.[18]

We have chosen Semliki Forest Virus as a model for the study of arbovirus replication and genetics. This introduction reviews the morphology, replication and genetics of arboviruses.

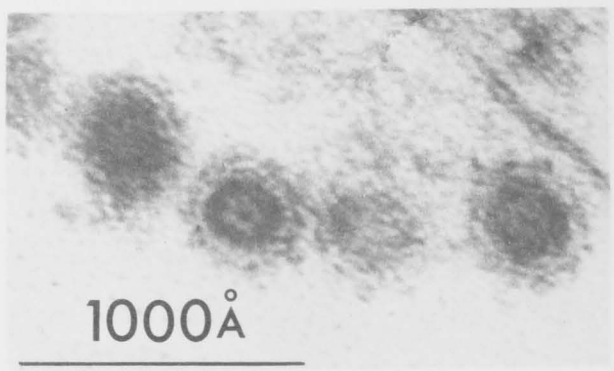
The Arboviruses

Arboviruses are defined as viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropod after a period of extrinsic incubation.[95] Arboviruses are divided into subgroups e.g. group A, group B and others. Such a classification of viruses based solely on ecological considerations is entirely different from universal systems of classification of viruses based on properties of the virions (i.e. type of nucleic acid, presence or absence of an envelope, symmetry, shape, size, etc.)

A number of arboviruses have been characterized physico-biochemically and Fenner (1968) proposed the name 'ENCEPHALOVIRUSES' for these viruses to distinguish them from the other arboviruses. Studies on six encephaloviruses, which belong to four antigenic sub-groups of group A in the arbovirus definition,[12] are reviewed below. These are Semliki Forest virus (SFV), Sindbis virus, Chikungunya virus and three equine encephalitis viruses namely, Eastern - (EEE), Western - (WEE) and Venezuelan - (VEE). These six viruses will be referred to collectively as 'encephaloviruses' in the following text.

Figure 1-1

SFV virions negatively stained with sodium silicotungstate.



SFV virions in thin section, stained with uranyl acetate and lead citrate. (Note: spikes of envelope are poorly stained)

Schematic diagram of SFV virion

Morphology and size of viruses

All encephaloviruses are spherical enveloped viruses bearing surface projections or 'spikes' (figure 1-1). No substructures were seen in negatively stained specimens. SFV, Sindbis virus and Middleburg virus (another encephalovirus) prefixed with formaldehyde before negative staining, have an appearance highly suggestive of cubic symmetry of the internal component.[77]

Encephaloviruses are small, measuring 400A-500A in diameter excluding the spikes.[61] Studies of thin sections of fixed viruses revealed the presence of a polygonal internal component (or 'nucleocapsid') measuring 260A-300A in diameter.[78] The nucleocapsid of Sindbis virus is electron-dense while those of SFV and Middleburg virus have an electron-lucent center which contains a centrally placed tiny electron-dense particle measuring 85A-125A in diameter (figure 1-1). Simpson and Hauser (1968b) suggested that the tiny electron dense particle may be the terminal end of a wound nucleocapsid component. No definite substructures were seen on the nucleocapsid.

The nucleocapsid is separated from the envelope by a narrow space which in SFV is 20A wide.[3] The envelope, which is of unit membrane structure, is 70A-75A thick in SFV and the spikes are about 110A long.[3]

Chemical composition of virus

Encephaloviruses contain RNA, protein and lipid and in EEE, some carbohydrate.

Viral RNA

The RNA is single-stranded and the base ratio has been determined for SFV, Sindbis virus and WEE and was found to be similar.[36,69,84] The bases Uracil, Guanine, Adenine and Cytosine are present in the ratios 20-23 : 26-27 : 27-30 : 24-26. The guanine content of WEE is slightly less, being only 22.3. Viral RNA is synthesised *de novo* in infected cells and its base ratio is different from that of cellular RNA.[69]

The reported RNA content is 5.8 per cent in Sindbis virus,[68] 4.4 per cent in EEE,[90] and 6.2 per cent in VEE.[93] These figures were obtained with viruses which have not been exhaustively purified and are unlikely to represent the true content.

Viral proteins

Viral proteins, synthesisd *de novo* in infected cells, constitute 49.1 per cent of EEE virion[90] and 65.5 per cent of Sindbis virion.[67,68] Only two major proteins are present in purified Sindbis[85,86] and WEE virions;[83] the membrane protein and the nucleocapsid protein. There are about 3-4 times as much membrane protein as nucleocapsid protein. The reported molecular weight, determined by polyacrylamide gel electrophoresis, of the membrane protein of Sindbis virus is 53,000 daltons and that of the nucleocapsid is 23,000 daltons.[86] In addition to these two major structural proteins, a third and minor protein is present in the nucleocapsid of SFV.[37] This protein migrated slower than the membrane protein in polyacrylamide gel electrophoresis; presumably having

a molecular weight greater than that of the membrane protein. The molecular weights of the membrane and nucleocapsid proteins of SFV are 50,000 and 35,000 daltons respectively.[44]

Viral lipid

The lipid content of WEE[84] and EEE[90] are 50 per cent and 54 per cent respectively, but in Sindbis[68] and VEE[93] it is lower, being only 44 per cent and 29 per cent respectively. With VEE the lipid content varied with different methods of purification.[93] The major constituents of lipid are phospholipid and cholesterol present in the ratio 3:1 in Sindbis virus and EEE. Small quantities of fatty acids were detected in EEE.[90] An analysis of the phospholipid, which is derived from the host cell, of Sindbis virus revealed the presence of Sphingomyelin, Lecithin and Phosphatidyl-ethanolamine in the ratio 1:1.5:1 which is different from that found in the host cell.[68]

Most, if not all, of the lipid is associated with the viral envelope.[84]

Carbohydrate

About four per cent of carbohydrate was detected in EEE.[90] The significance of its presence is not known. Carbohydrate was not tested for in other encephaloviruses.

Biological properties of viral components

Viral RNA

Infectious RNA, located in the nucleocapsid, has been extracted from encephaloviruses using sodium dodecyl sulphate (SDS) or phenol or a combination of SDS and phenol. The RNA is susceptible to ribonuclease treatment, indicating its single-stranded nature.[82]

Viral nucleocapsid

Nucleocapsids extracted from SFV[39] Sindbis virus[11,96] and WEE[83] were unable to form plaques in tissue culture but contained infectious RNA. Thus it seems that the viral envelope is essential for initiating plaque formation. Nucleocapsids lack hemagglutinating activity and viral lipid.[83]

Viral envelope

All encephaloviruses will hemagglutinate (HA) goose red blood cells; this activity is located on the envelope. Careful enzymic degradation of the envelope of SFV by Osterrieth[63] demonstrated that the HA activity resided in the spikes. The degraded virions, lacking spikes as observed in the electron microscope, lost all HA activity but retained full infectivity. Thus the spikes play no part in the infectivity process.

Viral growth in tissue culture

Outside their arthropod and vertebrate hosts, encephaloviruses have been grown in a large number of tissue culture systems (at least 14 cell types [61]). However biochemical studies of viral replication have mainly been confined to primary chick embryo cell monolayer cultures.

The growth cycle of encephaloviruses may conveniently be divided into the following steps: adsorption, penetration, eclipse and the synthesis and release of progeny virions.

Adsorption

Among the encephaloviruses, the adsorption of only SFV has been investigated in any detail.[63] Under ideal conditions, e.g. neutral pH, low NaCl concentration, high temperature (36^{o}) and the virus in a minimal volume of inoculum, the efficiency of adsorption was about 90 per cent of the theoretical value. Deviations from these conditions resulted in lower efficiencies of adsorption. The spikes of the envelope play no part in adsorption, only the membrane is essential.

Not all the adsorbed virions penetrate the cell successfully. A significant fraction of virions, which cannot be washed off the cells at the end of the adsorption period, was found to elute into the growth medium during the eclipse period.[9] When adsorption and penetration were successful, the virus was not neutralisable with specific antiserum.[63]

Penetration

SFV seemed to be taken into cells by phagocytosis but the actual penetration of individual virions from the phagocytic vacuoles into the cytoplasm has not been observed.[43]

Only a fraction of penetrated virions is uncoated. Experiments with ^{3}H uridine labeled purified SFV demonstrated that even at two hours after infection a significant amount of viral RNA extracted from the cytoplasm of infected cells probably came from uneclipsed virions.[41]

The eclipse, synthesis and release of virus

The eclipse period of encephaloviruses varies from 2-5 hours, being usually 3-4 hours and is dependent on the multiplicity of infection and the cell system. The eclipse periods of SFV at a multiplicity of infection of 20 and 80 PFU per cell were 3.5 hours and 2.5 hours respectively, and maximum viral yields were obtained at eight hours and 5.5 hours respectively.[58] The earlier production of virus at a high multiplicity of infection probably resulted from synchronization of multiplication. VEE in L cells has an eclipse period of 3-4 hours, of 4-5 hours in KB cells and maximum viral yield in L cells was obtained at 16-18 hours; and in KB cells, at 11 hours.[61] The maximum yield of encephaloviruses is high, about 200-1000 PFU per cell for WEE, 2700 PFU per cell for VEE and 2000 PFU per cell for SFV.[3,61]

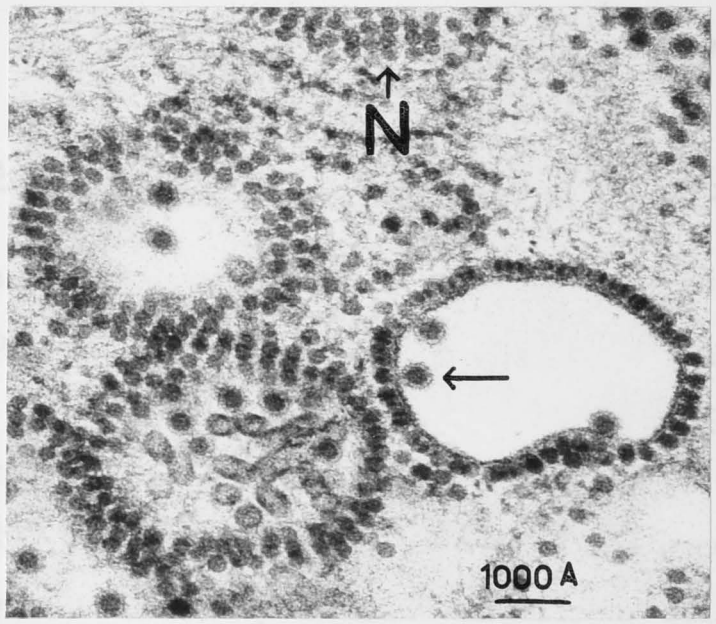


Figure 1-2

Electron micrograph of thin section of SFV infected cell

Electron microscopic studies of cells infected with Chikungunya virus,[14] WEE,[60] VEE,[62] Sindbis virus[74] and SFV,[3] revealed the presence of electron dense particles of the shape and size of the nucleocapsids of mature virions (N in figure 1-2). During the period of maximum viral production these particles were found scattered in the cytoplasm but often were associated with membrane structures; being found associated with ribosomes of the endoplasmic reticulum, or on, or near cell membranes or the membranes lining vacuoles. The viral nature of these particles was inferred when these particles were seen budding from cellular membranes to form virions (arrow, figure 1-2) of SFV, Chikungunya virus and WEE. WEE was also observed budding into vacuoles. These vacuoles may then migrate to the cell membrane and the virions released by a reverse process of phagocytosis. No budding virions were detected in cells infected with Sindbis virus or VEE. These two viruses presumably acquire an envelope at the cell membrane as they migrated through it.

Late in infection, aggregates of nucleocapsids, sometimes in paracrystalline arrays indicative of cubic symmetry,[48] were seen in cells infected with WEE, Sindbis virus and SFV, but not with VEE.

Recently, detail studies of cells infected with SFV[3,26,57] revealed the presence of rods or tubular structures and vesicles which are presumed to be virus-specific as they were absent in uninfected cells. Their significance is not known except that viral RNA synthesis seemed to be associated with the vesicles.[43]

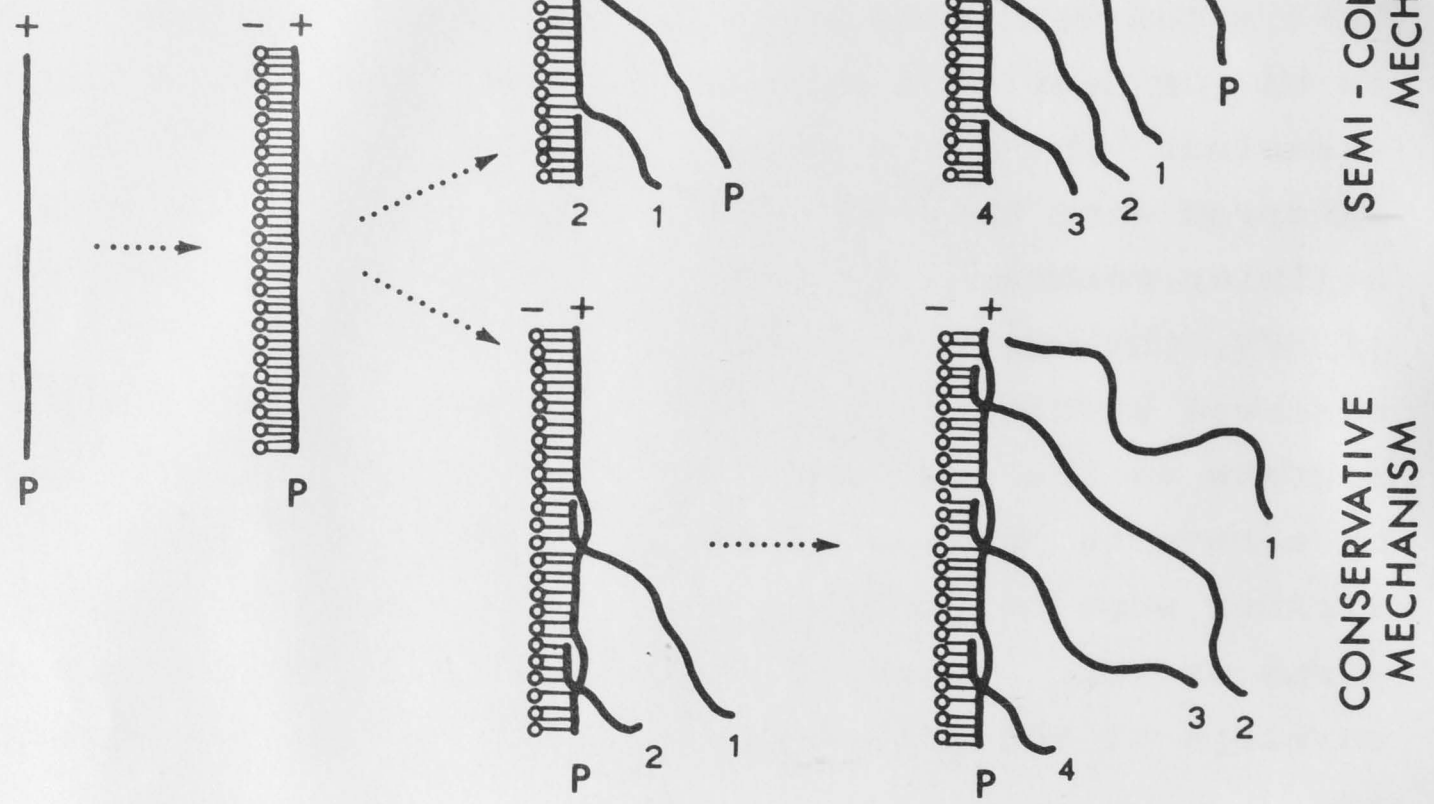


Figure 1-3. Mechanisms of viral RNA replication. 'P' is the input or parental RNA. 1,2,3 & 4 are the newly synthesised (progeny) RNA. See text for explanation.

Biochemical events in the viral growth cycle

The replication cycle can conveniently be discussed under 'early' events concerning viral RNA replication and 'late' events involving the synthesis of viral structural proteins, their assembly and the maturation of virions.

The early events - Replication of viral RNA

In cells, DNA is the information center and the synthesis of all RNA molecules is directed by DNA. The ability of a large number of riboviruses (containing single-stranded RNA) to multiply in cells in which DNA functions are suppressed (with Actinomycin D) indicates that cellular DNA doesnot play any intermediate part in the replication of these riboviruses which multiply in the cytoplasm.

The replication of single-stranded RNA has for a long time posed an intriguing problem. Elucidation of this problem progressed rapidly after the discovery of a double-stranded RNA in cells infected with the picornavirus, encephalomyocarditis virus.[59] This double-stranded RNA, suggested to be a 'replicative form' in RNA replication, has been demonstrated in all but one animal riboviruses investigated.[54] Recent intensive studies[54] of the replication of picornaviruses and encephaloviruses have suggested the replication scheme as shown in figure 1-3. The first step involves the synthesis of a complementary strand ('-' strand) using the input parental RNA ('+' strand) as a template.

TABLE 1-1. SEDIMENTATION COEFFICIENT OF VIRUS-SPECIFIC RNAs IN SUCROSE GRADIENT

RNA	Sedimentation Coefficient		
	SINDBIS [a]	SFV [b]	WEE [c]
VIRAL	40 S	42 S	40 S
INTERJACENT	26 S	26 S	26 S
RF	20 S	16 S	15 S
RI	NR	14-30 S	NR

a Yin and Lockart, 1968.

b Friedman, 1968b.

c Sreevalsan et al, 1968.

NR = not reported.

The '-' and '+' strands are held together by hydrogen bonds to form a double-stranded 'Replicative Form' (RF) RNA. In the second step the '-' strand of the RF serves as a template for the synthesis of progeny ('+' strands) RNA by one (or both?) of two mechanisms, the conservative and the semi-conservative mechanisms. The RF with nascent RNA of varying lengths attached to it is known as the 'Replicative Intermediate' (RI) RNA. Progeny RNA would either be incorporated into virus particles or be available for messenger RNA functions or be incorporated into further RF for the synthesis of more RNA.

Viral, RF and RI RNA have been extracted from the cytoplasm of cells infected with SFV,[36,58] Sindbis virus[10] and WEE.[84] In addition, an 'Interjacent' RNA[54] which sediments between viral and RF RNA in a sucrose gradient, has been extracted. Interjacent RNA is absent in the picornavirus system. Virus-specific RNAs can be differentiated by their sedimentation coefficients which are presented in Table 1-1.

Replicative Form and Replicative Intermediate RNA

The RI has a polydisperse profile (Table 1-1, presumably resulting from the presence of varying lengths of nascent RNA attached to the RF template (figure 1-3). Treatment of the RI of SFV with ribonuclease before sedimentation on a sucrose gradient resulted in a change in sedimentation coefficient to 16S indicating that the core of the RI is double-stranded RF RNA. When cells infected with SFV were

pulsed with ^{3}H uridine early in infection[36] or were pulsed very briefly during the phase of maximum viral production,[41] radioactivity was associated with the RI and RF. That the RI and RF are the first RNAs to be synthesised is predicted by the base-pairing mechanism of RNA replication.

Viral RNA and Interjacent RNA

The function of Interjacent RNA is not known. It may be a viral messenger RNA,[54] but available evidences suggest that it is viral RNA in a different configuration.[40,84] Both SFV viral and Interjacent RNA have the same base ratios and buoyant density in caesium sulphate.[39] Heat denatured viral RNA sediments at 26S.[40] Viral and Interjacent RNA of WEE[84] are interconvertible by dialysis against solutions of low or high ionic strengths respectively. Alternatively, viral RNA could be labile and breaks easily into two equal strands sedimenting as Interjacent RNA.[40,84]

Enzymes involved in RNA replication

Two enzymes or two enzyme functions may be required; one for the synthesis of double-stranded RNA and the other for the synthesis of single-stranded RNA from the double-stranded template.[54] This is a novel enzyme(s), a RNA-dependent RNA polymerase(s). This enzyme(s) extracted from cells infected with encephalomyocarditis virus, catalysed the synthesis *in vitro* of both double- and single-stranded RNA.[23] Evidence has been obtained that two enzyme functions may be required for the

synthesis of poliovirus RNA (Stanchek and Cooper, personal communication). Of the encephaloviruses, the RNA polymerase of SFV only has been studied.[56] However this enzyme catalysed the synthesis of double-stranded RNA only.

Interferon, puromycin and cycloheximide which are protein synthesis inhibitors, will block the synthesis of double-stranded RNA, thus providing indirect evidence that at least one enzyme is required for viral RNA synthesis.[40,70]

Site of viral RNA and RNA polymerase synthesis

When the cytoplasmic extract of cells infected with SFV was fractionated by sedimentation at various velocities, most of the polymerase activity and virus-specific RNAs were found in a large particle fraction sedimenting at 8,500 xg for 20 minutes.[56] Electron microscopic and detergent treatment studies of this fraction indicated that both polymerase and RNA were associated with membrane structures.[39]

Late events of the growth cycle

This half of the growth cycle was investigated only recently. Subsequent to RNA synthesis, structural proteins are synthesised. Structural proteins and RNA are then assembled and mature virions released.

Synthesis of viral structural proteins

When infected cells incubated in the presence of radioactive amino-acids were disrupted and the

cytoplasmic fraction solubilised and subjected to electrophoresis in polyacrylamide gel, six peaks of radioactivity (proteins) were reported for SFV,[38,44] and 10-12 for Sindbis virus.[86] Only the structural proteins (two in Sindbis, three in SFV) have been identified. It is not known if some of the proteins were cleavage products of large proteins as was reported for poliovirus.[47]

(a) Nucleocapsid protein

Pulse-chase experiments with cells infected with WEE suggested that nucleocapsids are precursors of mature virions.[83] Radioactivity was chased from intracellular nucleocapsids to extracellular virions. Also, the synthesis of nucleocapsids preceded the synthesis of virions. The precursor role of nucleocapsids is consistent with the findings of electron microscopic studies.

(b) Membrane protein

The membrane protein of Sindbis virus is synthesised de novo[67] but the phospholipid component of the envelope is derived mainly from the host cell.[69] Synthesis of the membrane precedes the synthesis of mature virions.[10] Late in infection virus-specific proteins constitute 7-8 per cent of the total protein of infected cells, of which the membrane protein is the major component, representing three per cent of the total cellular protein.[86]

Site of viral protein synthesis

Like the synthesis of viral RNA, viral protein synthesis (more than 50 per cent) is membrane bound.[35]

In contrast, only about 15 per cent of protein synthesis in uninfected cells appeared to be membrane bound.

Nucleocapsids of SFV are assembled extremely rapidly, within three minutes. As viral RNA synthesis requires ten minutes, this means that a pool of viral RNA must be present from which nucleocapsids encapsulate their RNA complement.[35] Nucleocapsids of Sindbis virus require 10-15 minutes for formation.[7] This rapid formation of nucleocapsids requires viral RNA and protein synthesis in close juxtaposition, e.g. on membrane structures.

In summary, the replication cycle of encephaloviruses is as follows: input parental RNA is converted to a double-stranded form which serves as a template for the synthesis of progeny RNA. Viral RNA probably also acts as a messenger for the synthesis of structural proteins. Viral RNA is then incorporated into nucleocapsids which then acquire an envelope at cellular membranes to form mature virions.

Virus-cell interactions

Both SFV[89] and Sindbis virus[69] inhibit host cell RNA and protein synthesis only late in the growth cycle when the bulk of the virus is produced. Substantial inhibition of cellular phospholipid synthesis, from one-third to half that in uninfected cells, was reported for Sindbis virus.[69] The effect of infection on the metabolism of cellular DNA has not been studied.

The cells respond to infection by producing interferon. Chick embryo cells infected with SFV produced little interferon during the first cycle of viral growth. During the second cycle of viral growth massive and inhibitory quantities of interferon were produced.[89] The inducer of interferon production has not been identified but must be some early viral protein(s) as viral RNA replication is not essential for interferon production.[51,81]

Conditional Lethal Mutants in the study of viral functions and genetics

An elegant method of studying viral functions and genetics is in the use of 'conditional lethal mutants' which are either normal or defective in growth under conditions imposed by the experimenter. Two classes of conditional lethal mutants have been investigated: 'amber' mutants which grow in permissive hosts but not in non-permissive hosts, and 'temperature-sensitive' or *ts* mutants which replicate normally at a low (permissive) temperature but not at a high (non-permissive or restrictive) temperature. In both types of mutation the wild-type virus, the parental strain from which the mutants were derived, grows normally under both permissive and restrictive conditions. Conditional lethal mutants are easy to select for since it is not necessary to know what function is altered. Theoretically, such mutations can occur in any part of the genome.

Epstein *et al* (1963) exploiting both amber and *ts* mutants, were able to map the T_4 genome and characterize

the gene products. Fenner in 1965 pointed out the potential usefulness of conditional lethal mutants in the study of animal viruses. However only ts mutants are readily obtainable and studies of such mutants have been reported for rabbit pox, polyoma, polio, foot-and-mouth disease, Newcastle disease and reo-viruses and the encephaloviruses: SFV and Sindbis virus.[31]

Temperature-sensitive mutants of animal viruses

At the molecular level, the ts defect results from a change in the base sequence of the nucleic acid. With point mutation, one base is substituted resulting in the synthesis of a novel amino-acid during protein synthesis. The resulting protein may function normally at the permissive temperature. At the restrictive temperature this protein, if it is an essential viral protein, may be unable to assume or maintain a functional configuration resulting in a loss of biological activity. The presence of altered bases in the nucleic acid was inferred by Wittman and Wittman-Liebold[94] who demonstrated that several ts mutants of Tobacco mosaic virus had changes in the amino-acid sequence of the viral coat protein when compared with wild-type virus.

Thus, ts mutants are 'mis-sense' mutants, mutants in which the altered nucleic acid is read and altered gene products made. Amber mutants are 'non-sense' mutants, in which the altered base results in a non-sense codon (UAA or UGA or UAG) causing premature release of the protein being synthesised.

Some of the consequences resulting from the nature of ts mutation are:

(a) mutants may not grow as efficiently as wild-type virus at the permissive temperature as the novel amino-acid may not function as efficiently;

(b) the gene products may exhibit altered heat stability;

(c) there may be a change of antigenicity if the mutation affected the antigenic determinants or configuration of the protein which is of antigenic significance;

(d) the mutants tend to revert to the wild-type character with a relatively high frequency; and

(e) the mutants are 'leaky', i.e. functional activity is found at the restrictive temperature. Thus large numbers of mutants must be isolated to obtain a few which are stable enough for physiological and genetic studies.

Temperature-sensitive mutants, being point mutation mutants, should be extremely useful for the study of viral functions and genetics. They offer a means for 'total mapping'[18] of small riboviruses whose small genome is not expected to code for more than 12-15 polypeptides. Genetic mapping of two animal riboviruses has begun and preliminary maps have been constructed for ts mutants of poliovirus[20] and influenza virus.[53]

Temperature-sensitive mutants of encephaloviruses

ts mutants have been isolated from SFV and Sindbis virus at about the same time but independently by Sambrook (1965) and by Burge and Pfefferkorn (1966a).

ts mutants of Sindbis virus

Burge and Pfefferkorn chose for their study a genetically stable heat resistant (HR) strain derived from the wild-type virus.[8] Twenty-three ts mutants were obtained from this HR 'wild-type' by treatment with ethylethane sulphonate, nitrous acid and nitrosoquanidine. These mutants were defective in growth at 39^{o}-40^{o} but not at 27^{o}. Physiologically, these mutants were placed in two groups: the RNA^{-} and the RNA^{+} mutants which were unable or able, respectively, to make viral RNA at the restrictive temperature.

RNA^{-} mutants were found to be defective in an early function of viral growth, presumably the synthesis of viral RNA polymerase so that the input parental RNA was not converted to the RF which is an essential step in RNA replication (figure 1-3). Virions of these mutants, produced at 27^{o}, were found to be as heat stable as the HR wild-type, indicating normal structural proteins in the virions.[8]

RNA^{+} mutants were found to be defective in late functions involving the formation of structural proteins, or some maturation steps. Yin and Lockart (1968) studying three mutants supplied by Pfefferkorn, arrived at the same results. The virions of most RNA^{+} mutants were heat labile and probably contain altered structural

Table 1-2. Functional defects of ts mutants of Sindbis virus

ts MUTANT	COMPLEMENTATION GROUP	RNA SYNTHESIS AT 40°	NUCLEOCAPSID FORMATION AT 40°	HEMADSORPTION AT 40°	HEAT STABILITY AT 60°	PRESUMED DEFECT IN:
4,17,19 21,11	A	-	-	-	+	an enzyme for RNA synthesis
6	B (?)	-	-	-	+	another enzyme for RNA synthesis (?)
2,5,13	C	+	-	+	-	a nucleocapsid protein
10,23	D	+	+	-	-	an envelope protein
20	E	+	+	+	+	a maturation protein (?)
9	fails to complement	+	+	-	-	?

- = defective or heat labile, + = not defective or heat stable.

proteins. One RNA^+ mutant was heat stable and is thought to be defective in a maturation step so that once formed, the virions were heat stable.[11] Table 1-2 summarises the results obtained with ts mutants of Sindbis virus.

Genetic studies (Recombination and Complementation)

Recombination was not demonstrated although the conditions were ideal i.e. crossing mutants from two different cistrons (RNA^+ and RNA^-), and sensitive i.e. any recombination of the order of 10^{-4} could easily be detected as the mutants have low reversion frequencies of less than 10^{-5}.[9]

Complementation, a test which allocates complementing mutants to different cistrons, was demonstrated for all pairs of RNA^- x RNA^+ mutants and for certain pairs of RNA^- x RNA^- and RNA^+ x RNA^+ mutants.[9] RNA^- mutants were placed in two complementation groups (here, complementation probably resulted from highly efficient intracistronic complementation). RNA^+ mutants fell into three complementation groups (intercistronic complementation) (Table 1-2).

The notable features of complementation were:

(a) the multiplicity of infection was not important in the pair RNA^+ x RNA^+ but was critical when one parent was RNA^-. Increasing the multiplicity of infection of the RNA^- parent in the pair RNA^+ x RNA^- increased the efficiency of complementation;

(b) in complementation between RNA^- x RNA^+, the RNA^+ phenotype was preferentially represented in the progeny;

(c) although the efficiency of complementation was high, from 5-200 fold increase in yield over the combined yields of the individual parents, the absolute efficiency was low, being only 1-3 per cent of wild-type yield under similar conditions;

(d) with high input multiplicities, complementation proceeded with the same kinetics as the growth of wild-type virus; and,

(e) most importantly, complementation resulted from new biosynthesis and not by reassortment of the input parental viral materials.

Grouping of the mutants by both genetical and physiological tests was in complete agreement i.e. all the mutants in the same complementation group have the same defect.[66]

Recently, Sindbis virus ts mutants were used to study the induction of interferon production.[51]

ts mutants of Semliki Forest virus

Nine ts mutants were isolated by Sambrook (1965) after growing SFV in the presence of 5-fluorouracil. These mutants were defective at 38.3° but replicated normally at 28°. At intermediate temperatures mutant viruses were produced and a 99 per cent inhibition ('cut-off') of yield occurred usually at 36°-38°. Plaque formation of wild-type virus and mutants were inhibited by interferon. All the mutants and wild-type virus induced interferon production at the restrictive temperature and the amount of interferon produced was

related to the ability of the virus to grow at the restrictive temperature, i.e. mutants with high leak rates induced more interferon production than mutants with low leak rates.

Both recombination and complementation were demonstrable for certain pairs of mutants. But the results were not easily reproducible. Recombination was difficult to demonstrate under standard conditions, i.e. by crossing at the permissive temperature, but was easier to detect when mixed infection was carried out at the restrictive temperature. The results of complementation and recombination did not allow the mutants to be placed in groups.

The study of SFV ts mutants was extended by the present author. A further suite of 29 ts mutants were isolated and these together with Sambrook's mutants were studied in detail to gain an understanding of the replication and genetics of Semliki Forest virus.

CHAPTER 2

MATERIALS AND METHODS

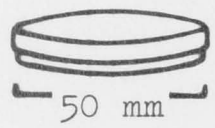
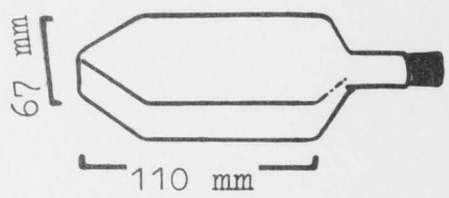
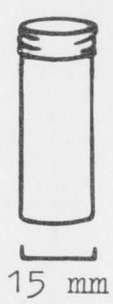
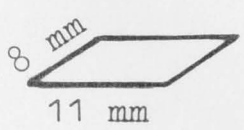
Monolayers formed in	Volume of growth medium	No. of cells per confluent monolayer[a]
 Petri-dish [monolayer]	5 ml	7.5×10^6
 'T' flask [large monolayer]	15 ml	2.5×10^7
 Screw-capped bottle [small monolayer or Minilayer]	1 ml	7×10^5
 Cover-slip [non-confluent monolayer]	5 ml[b]	$5\text{-}10 \times 10^4$

Figure 2-1. Monolayer culture of chick-embryo cells.

a. Determined after 24 hr incubation at 38°.
b. Cover-slips were contained in petri-dishes.

Cells

Chick embryo cell (CE) monolayers were used in all experiments. Ten day old chick embryos were minced with scissors and treated with 0.25 per cent trypsin for 20 minutes at 37°. The resulting cell suspension was centrifuged to remove trypsin and the cells were suspended in growth medium containing five per cent calf serum and filtered through cotton gauze to obtain a single cell suspension. Appropriate numbers of cells were seeded in culture containers to give a confluent monolayer after about 20 hours incubation at 38°. Details of culture containers etc. are given in figure 2-1.

Media

(a) Growth medium was prepared by supplementing Earle's saline with 0.1 per cent bovine serum albumin fraction V, 0.1 per cent yeast extract, 0.5 per cent lactalbumin hydrolysate and 0.1 per cent sodium bicarbonate to adjust the pH to 7.4. This medium was used for the growth of both cells and virus and for diluting virus (Cooper 1967b).

(b) Agar overlay for plaque assay of infectivity consisted of growth medium containing 0.75 per cent agar, 100 µg per ml DEAE Dextran, 0.05 per cent sodium bicarbonate and was buffered with 0.002 per cent Tris-(hydroxymethyl)aminomethane (= Tris) pH 7.4 (Cooper, 1967b). In later experiments dextran was omitted and agarose replaced the agar as plaques formed under agarose were larger and clearer.

(c) Phosphate buffered saline (PBS) was prepared according to Dulbecco and Vogt (1954).

(d) Gelatin saline consisted of 0.5 per cent gelatin in Calcium-Magnesium saline (Fazekas, Graham and Jack, 1958).

Virus

Semliki Forest virus strain 25639 was obtained from the Rockefeller Virus Laboratories and cloned by picking a single plaque formed in CE monolayer infected at 38.3°. The plaque was picked by removing the plug of agar overlying the plaque with the broad end of a pasteur pipette. The agar plug, with the cells of the plaque attached to it, was placed in 0.5 ml of growth medium to elute the virus. A stock was prepared by growing the virus in CE monolayers at 38.3° in the presence of 0.1 µg per ml of actinomycin D (AMD) for about 20 hours.

Chemical mutagenesis of SFV

(a) Mutation with 5-fluorouracil (5-FU)

SFV was grown in CE monolayers in the presence of 5 mM of 5-FU in Eagle's medium lacking calf serum for 24 hours (Sambrook, 1965).

(b) Mutation by hydroxylamine (HA)

SFV was treated with 1.0 M HA in 1.3 M NaCl and 0.025 M phosphate buffer pH 7.6 (Thiry, 1963). After 20 minutes at 37°, the virus was diluted 200 fold in growth medium.

(c) Mutation by N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

SFV was treated with 0.1 mg per ml of NTG in 0.01 M Tris pH 7.6 for 10, 20 or 30 minutes at room temperature (Burge and Pfefferkorn, 1966a). The virus was then diluted 10 fold in cold growth medium followed by overnight dialysis against cold growth medium to remove NTG. The treated virus was stored at -70°.

Isolation of ts mutants

The restrictive and permissive temperatures for SFV ts mutants are 38.3° and 28° respectively (Sambrook, 1965). In some experiments 38.5° and 30° were used.

Mutagen treated virus was diluted in growth medium and CE monolayers were infected with about 5 PFU per monolayer. After three days incubation at 28°, well isolated plaques were picked and the virus eluted into growth medium and assayed at both 38.3° and 28°. If the titer (PFU) at 38.3° was less than one per cent of that at 28°, the virus was considered as a presumptive ts mutant and a stock was prepared by two cycles of growth at 28°. Stocks of presumptive ts mutants were tested for two properties:

(i) efficiency of plating at 38.3° (e.o.p. at 38.3°), i.e. ratio of titer assayed at 38.3° to titer assayed at 28° which is a measure of the wild-type content (from reversion) of the stock; and

(ii) leakiness of yield at 38.3°, i.e. ratio of yield in a one-step growth cycle at 38.3° to that at 28°. If the e.o.p. and leak yield at 38.3° of presumptive ts mutant stocks were 10^{-4} and 10^{-3} or less, respectively, they were accepted for further characterization.

Method of infecting cells and the growth of virus in liquid medium

Preformed CE monolayers were inoculated with 10-15 PFU per cell and kept at 4° for 60 minutes followed by overnight incubation at 4° in growth medium containing 2.5 per cent calf serum and 1 µg per ml AMD to inhibit cellular RNA and interferon synthesis. The following morning fresh growth medium containing 2.5 per cent calf serum and 1 µg per ml AMD (unless otherwise stated) was supplied and the cells incubated at the desired temperature (Taylor, 1965); this time was taken as time zero of the growth cycle.

In all experiments infected cells were incubated in the dark as SFV is light sensitive (Appleyard, 1967).

Infectivity assays

(a) Infectious virus

Preformed monolayers in 50 mm petri dishes were inoculated with 0.1 ml of virus diluted in growth medium. After a 1-2 hour adsorption period at room temperature in the dark, the monolayers were overlaid with 5 ml of agar (or agarose) overlay. After 1 day incubation, 1.5 ml of agar overlay

containing 0.015 per cent neutral red was added to stain the cells. Plaques were counted after 2 days at 38.3° or after 3 days at 28°.

(b) Infectious RNA

CE monolayers were washed with PBS containing 1 mg per ml of bentonite, followed by a wash with 0.4 M NaCl in 0.1 M Tris pH 8.2 and containing 100 µg per ml DEAE Dextran. Each monolayer was inoculated with 0.1 ml of RNA diluted in 1 M NaCl in 0.1 M Tris pH 8.2 (Appleyard, 1967). After 15 minutes the monolayers were overlaid with agarose overlay and plaques were counted after 2 days.

Temperature control

Strict temperature control was necessary in experimenting with ts mutants. For all experiments involving viral replication, water-baths maintained at the desired temperature (± 0.05°C) by Braun 'Thermomix' heaters and thermostats (Melsungen, Germany) were used. 'T' flasks and petri-dishes sealed in plastic boxes were completely immersed in the water. Screw-capped bottles were incubated in water-baths by hanging them from polystyrene holders floating on the water. For plaque assay of virus at temperatures above 30°, petri-dishes were packed in plastic boxes which were sealed with snap-on lids and completely immersed in water. For plaque assay at 28° to 30° infected cells were incubated in standard water-jacketed incubators.

Viral RNA synthesis

CE minilayers were infected as described and incubated at 38.3° in the presence of AMD. After 2 hours at 38.3° the medium was replaced by fresh prewarmed medium lacking calf serum and containing AMD and 2 μC of ^{3}H uridine per ml. After 6.5 hours, which is the time of maximum viral RNA synthesis (see Chapter 4), the cells were washed twice with cold PBS and lysed with 0.3 N NH_4OH. Trichloroacetic acid was added to a final concentration of 10 per cent and the precipitate formed was collected, dissolved in 1.2 N NH_4OH, dried on filter paper and counted for radioactivity as described below.

Extraction and sucrose gradient analysis of viral RNA

Infected CE monolayers (in 'T' flasks) were labelled with 30 μC of ^{3}H uridine per monolayer from 2-6.5 hours at 38.3°. RNA was extracted from the cytoplasm of infected cells with sodium dodecyl sulphate (SDS) and phenol according to the method of Mecs et al (1967). Sedimentation of RNA on a 6-30 per cent sucrose gradient (prepared in Tris buffer: 0.1 M KCl, 0.01 M Tris pH 7.2 and 0.001 M disodium ethylenediaminetetracetate (EDTA)) was for 2.5 hours at 38,500 rpm in a SW 39 rotor. Gradient fractions were collected on filter paper strips (Whatman, no. 541), dried, washed in perchloric acid and counted in a toluene base scintillation fluid as described by Dalgarno et al (1966) in a Packard Tri-Carb liquid scintillation counter.

Extraction and sucrose gradient analysis of viral nucleocapsids

Monolayers grown in 'T' flasks and infected as described, were each labelled with 40 μC of ^{3}H uridine

from 2-7.5 hours when incubated at 38.5° and from 2-9 hours when incubated at 30° (viral growth is slower at 30°).

In some experiments cells were labelled with 30 µ C of ^{14}C reconstituted protein hydrolysate. The cells were washed twice with cold PBS (lacking Ca^{++} and Mg^{++}), scraped off the glass, pelleted and resuspended in 0.5 ml buffer (0.01 M KCl, 0.00015 M $MgsO_4$, 0.01 M tris pH 7.4 - Yin and Lockart, 1968). The cells were disrupted in a small dounce homogeniser after swelling for 15 minutes in the above hypotonic buffer. Nuclei and cellular debris were removed by centrifugation at 600 xg for 10 minutes (Friedman and Berezesky, 1967). Nonidet P40 (Friedman, 1968a) and ribonuclease were added to the cytoplasmic extract to give final concentrations of 0.1 per cent and 0.1 µg per ml respectively and kept at room temperature for 10 minutes. The preparation was then centrifuged in a 15-30 per cent sucrose gradient (prepared in the above buffer) for 60 minutes at 38,000 rpm in a SW 39 rotor at 4°. Gradient fractions were collected and counted for radioactivity as described. Purified chick liver ribosomes were used as a 74S optical density marker.

Hemadsorption

The hemadsorption method of Burge and Pfefferkorn (1967) employing ^{51}Cr-labelled gander cells was adopted with slight modifications; gander cells were labelled with 0.1 mC per ml of Sodium chromate ($^{51}CrO_4^{2-}$) and 0.2 ml of a 4 per cent suspension of labelled cells in borate-pyrophosphate buffer pH 6.5 was inoculated onto an infected CE monolayer of 8.5×10^6 cells. This

hemadsorption test was used to detect the presence of viral membrane protein formed on the surface of infected cells.

Hemagglutination

Hemagglutination (HA) of acetone extracted virion antigen was performed according to the method of Clarke and Casals (1958) with gander red blood cells at a final pH of 6.4. After a 1 hour incubation period at 37°, the HA titer was determined as the highest dilution of antigen in which a positive pattern was present.

Demonstration of viral antigen with fluorescent antibody

Non-confluent CE monolayers grown on cover-slips were infected at 4° for 90 minutes, washed and incubated at 30° or 38.5° in growth medium lacking AMD (AMD caused a high cytoplasmic fluorescence in uninfected cells). Eight hours after infection, the cells were washed with PBS, dried in air and fixed with acetone for 10 minutes. The cells were then treated with rabbit anti-SFV serum (extensively adsorbed with uninfected CE cells) for 1 hour at 38°, washed with PBS and stained with fluorescein labelled goat anti-rabbit globulin containing 1 per cent rhodamine for 20 minutes at 30°. After washing with PBS, the cells were examined with a UV fluorescent microscope.

Heat stability of viral infectivity

Virus was diluted with an equal volume of gelatin saline and 0.4 ml of the mixture was pipetted into a large tube immersed in a water-bath maintained at the

desired temperature ± 0.05°C. At various time intervals aliquots were removed, immediately diluted 10 fold in cold gelatin saline and residual infectivity was assayed at 30°.

Electron Microscopy

Cells were scraped off the glass, pelleted and fixed with 1 per cent gluteraldehyde (in PBS) for 5-10 minutes at room-temperature and postfixed with 1 per cent osmium tetroxide (in PBS) for 60-90 minutes at 4°. After washing with PBS containing 10 per cent sucrose, the cell pellet was dehydrated in acetone. All cell pellets were stained in a saturated solution of uranyl acetate in 70 per cent acetone for 30 minutes at room temperature. After dehydration in 100 per cent acetone, the cells were embedded in araldite (as instructed, Durcupan, ACM). Thin sections cut with a Reichert microtome using glass knives were mounted directly on 400 mesh grids. The sections were then stained with 0.2 per cent lead citrate in 0.1 N NaOH (Venable and Coggeshall, 1965) for 1 minute, washed and examined in a Philips EM 200 electron microscope.

For negative staining, a sample was applied onto a formvar or carbon coated grid and excess sample was removed with a piece of filter paper after 1 minute. 1 per cent sodium silicotungstate was applied on the grid and excess stain was removed after about 30 seconds (N.G. Wrigley, personal communication). The grid was dried in air and examined immediately.

Source of chemicals

Chemicals	Source
Actinomycin D	Gift from Merck, Sharp and Dohme, Rahway, New Jersey.
Araldite (durcupan, ACM)	Fluka AG, Switzerland.
DEAE Dextran	Pharmacia, Uppasala, Sweden.
Fluorescein conjugated goat anti-(rabbit serum IGG) globulin	Microbiological Associates Inc., Bethesda, Maryland.
5-Fluorouracil	Gift from Roche Products Ltd. to Dr P. D. Cooper.
Hydroxylamine hydrochloride	The British Drug Houses Ltd.
Nitrosoguanidine	K & K Labs. Inc., Plainview, N.Y.
Nonidet P40	Obtained from Dr E. M. Martin.
Ribonuclease (pancreatic)	Worthinton Biochem. Co., N.J.
Sucrose (ribonuclease-free)	Mann Research Labs. Inc., N.Y.

Radio-chemicals	Source
^{3}H-Adenosine (2.34 C/mM)	The Radiochemical Centre, Amersham, England.
^{3}H-Leucine (1.0 C/mM)	ditto
Sodium chromate ($^{51}CrO_4^{2-}$)	ditto
^{3}H-Uridine (20-28 C/mM)	ditto
C^{14} Reconstituted Protein Hydrolysate	Schwarz BioResearch, Inc., N.Y.

CHAPTER 3

THE ISOLATION AND GROWTH CHARACTERISTICS OF SEMLIKI FOREST VIRUS *ts* MUTANTS

INTRODUCTION

Semliki Forest virus possesses a number of properties advantageous for the study of viral genetics and physiology, namely, (i) it forms plaques readily in a number of cell lines, (ii) it has a rapid growth cycle in chick embryo cells and high titer stocks are easily prepared, (iii) it grows equally well over a wide range of temperature, facilitating the isolation of temperature-sensitive mutants, (iv) it has a high PFU to virus particle ratio of 1:5 (Osterrieth, 1966), and (v) it is a small ribovirus and its RNA of molecular weight of about 2×10^6 daltons is not expected to code for more than 12-15 polypeptides. Initial attempts by Sambrook (1965) to isolate *ts* mutants from SFV were highly successful. He obtained 9 *ts* mutants after growing SFV in the presence of 5-Fluorouracil and studied them in some detail.

Determination of the permissive and restrictive temperatures for the *ts* mutants was based on the lower and upper limits of the temperature range of normal growth and plaqueing efficiency of wild-type virus. Wild-type virus was grown over a range of temperature from 25^o to 40^o. In the absence of AMD, essentially normal yields were obtained from 28^o to 38.5^o. In the presence of 0.1 µg AMD per ml, a normal yield was obtained up to 39^o. Virtually the same plaqueing efficiency was obtained from 25^o to 38^o. On the basis of these 2 experiments, 28^o was chosen as the permissive temperature and 38.3^o the restrictive or non-permissive temperature. Although unable to grow at the restrictive temperature, the mutants produced some infectious virus

below that temperature and a 99 per cent inhibition ('cut-off') of normal yield occurred at temperatures between 34^{o} and 38^{o}.

This chapter describes the isolation of more ts mutants and a study of the growth characteristics of these and Sambrook's mutants. Sambrook's mutants have been renumbered as follows: ts-24 = ts-2, ts-4 = ts-3, ts-17 = ts-5, ts-14 = ts-6, ts-51 = ts-8, ts-13 = ts-9 and ts-23 = ts-10, 2 other mutants have not been accounted for. In some experiments the permissive and restrictive temperatures were increased to 30^{o} and 38.5^{o} respectively.

Table 3-1. Growth of SFV in the presence of AMD.

Overnight incubation at 4° in the presence of AMD at	Yield (PFU per ml) at 38° in the presence of AMD at		
	1 µg/ml	3 µg/ml	5 µg/ml
1 µg/ml	37×10^7	26×10^7	5×10^7
3 µg/ml	18×10^7	17×10^7	5×10^7
5 µg/ml	5×10^7	15×10^7	2×10^7

Table 3-2. Effect of AMD on SFV virions.

Sample	Titer, PFU/ml x 10^7, after incubation at room tempt. for				
	1hr	2hr	3hr	4hr	9hr
Virus without AMD	142	57	63	60	50
Virus with 1 µg AMD/ml	87	180	119	184	>200

RESULTS

Conditions for the growth of wild-type virus

Taylor (1965) reported that AMD did not inhibit SFV replication but inhibited interferon production in infected cells. Since AMD inhibits DNA-dependent RNA synthesis (Reich and Goldberg, 1964), it is likely that no host cell DNA mediated function is required for the replication of SFV. A convenient way of infecting cells and abolishing interferon production is by incubating the cells with virus in growth medium containing AMD at 4^{o} overnight, as described by Taylor (1965) (see Chapter 2). On warming the cells, virus multiplication commenced immediately. Experiments were set up to find the optimum concentration of AMD required for maximum virus production with our SFV-chick cell system.

Minilayers infected with 10-15 PFU per cell were divided into 3 groups. Each group was then incubated overnight at 4^{o} with 1, 3 or 5 µg AMD per ml. The following morning the cells were washed 3 times to remove unadsorbed virus and minilayers in each group were incubated at 38^{o} in the presence of 1, 3 or 5 µg AMD per ml. Calf serum at a concentration of 2.5 per cent was present throughout to preserve the integrity of the cells. After 7 hours at 38^{o} the cells were frozen, thawed and assayed for infectious virus. The results are presented in Table 3-1.

Maximum viral yield was obtained when cells were treated with 1 µg AMD per ml in both the overnight incubation at 4^{o} and growth at 38^{o}. This concentration

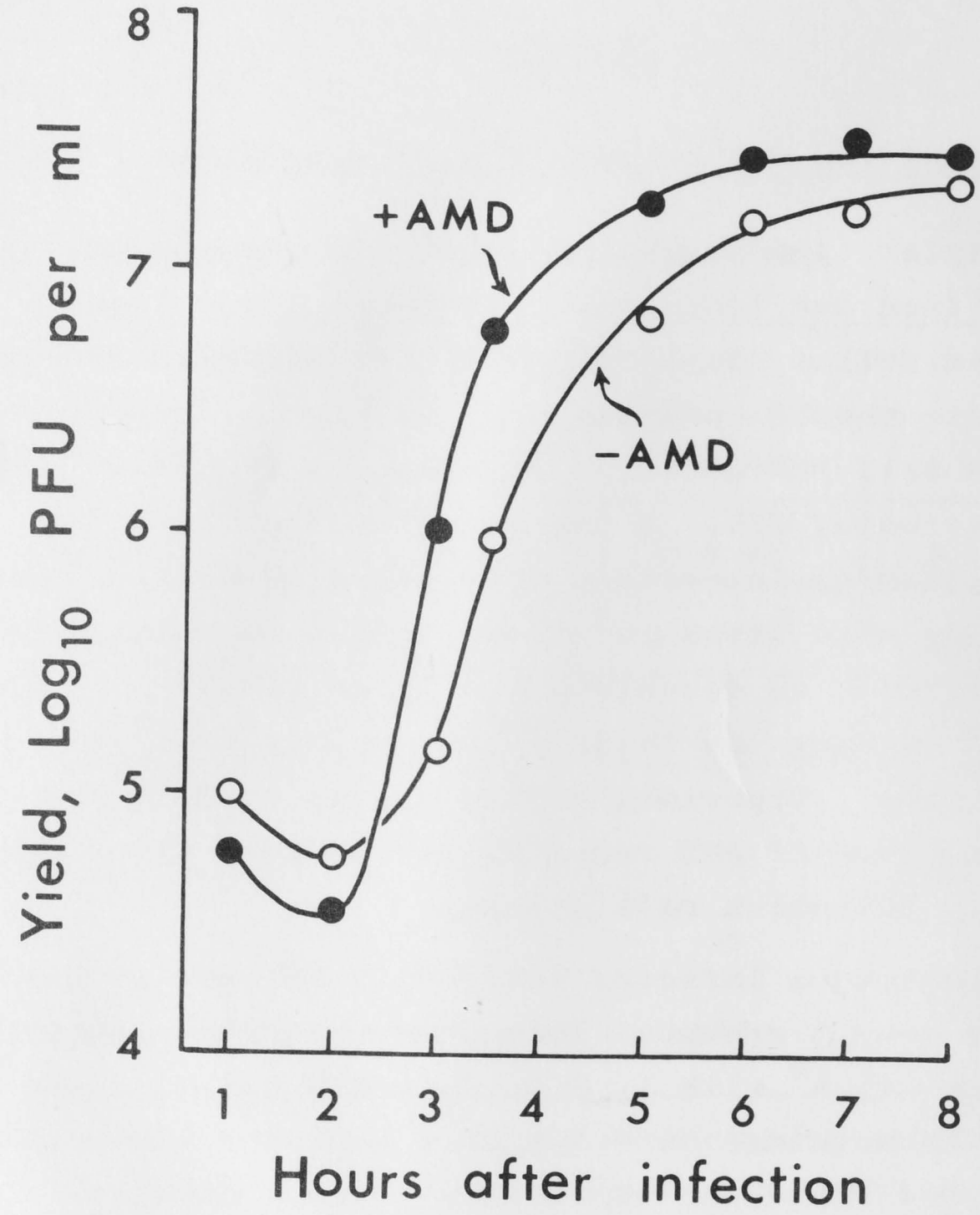


Figure 3-1. Growth curves of SFV in the absence and presence of AMD.

Infected minilayers were incubated at 38° in the absence or presence of 1 μg AMD per ml. Samples were at intervals, frozen, thawed and assayed for infectivity.

of AMD was used in most experiments involving viral growth in liquid growth medium. Higher concentrations of AMD probably kill the cells more rapidly resulting in lower yields of virus, as observed.

Effect of AMD on viral growth curves

When growth curves of wild-type virus grown in the absence or presence of 1 µg AMD per ml were examined, the latent period was much shorter, virus was released more rapidly and the final titer was higher in AMD treated cells (figure 3-1).

Effect of AMD on virions

The effect of AMD on virions was tested by incubating the virus suspension with 1 µg AMD per ml for up to 9 hours at room temperature in the dark. The treated virus was diluted and inoculated onto CE monolayers. After 30 minutes the monolayers were washed twice to remove AMD and unadsorbed virus and then overlaid with agar overlay. The titers of AMD treated samples were in most cases higher than those of untreated virus (Table 3-2). The reason for this difference in titer is not known

Chemical mutagenesis of SFV and the isolation of ts mutants

Mutagens were used to increase the mutation rate of SFV to make the isolation of large numbers of ts mutants feasible. The 3 mutagens used to induce ts mutants with SFV have been shown to be mutagenic for a number of

TABLE 3-3. CHEMICAL MUTAGENESIS OF SEMLIKI FOREST VIRUS

Mutagen[a]	Mode of action[b] on viral RNA	Duration of treatment	No. of ts mutants isolated	Isolation[c] rate	Inactivation of infectivity
5 - FU (5 mM)	substitutes Uracil,[1] pairs with G instead of A	24 hr	11	1.0 %	ND[d]
HA (1 M)	acts 'in vitro' on[1] Cytosine which then pairs with A instead of G	20 min	13	ND[d]	10^{-4} drop in titer
NTG (0.1 mg/ml)	deamination of bases?[2]	10 min	0	-	43 %
		20 min	1	0.7 %	76 %
		30 min	13	5.2 %	85 %

a. 5 - FU = 5-Fluorouracil, HA = Hydroxylamine
NTG = N-methyl-N'-nitro-N-nitrosoguanidine

b. A = adenine, G = guanine

c. $\frac{\text{no. of plaques recovered}}{\text{total no. of plaques tested}} \times 100$

d. ND = not determined

1. Freese, 1963.
2. Singer et al, 1968.

other animal viruses: 5-Fluorouracil was used to induce ts mutants with poliovirus (Cooper, 1964), Foot-and-mouth disease virus (Pringle, 1965), influenza virus (Simpson and Hirst, 1968; Mackenzie, 1968) and reovirus (Ikegami and Gomatos, 1968); ts mutants of polyoma virus (Eckart and DiMayorca, quoted by Fenner, 1969) and influenza virus (Mackenzie, 1968) were obtained with Hydroxylamine, and Nitrosoguanidine was used to induce ts mutants from Sindbis virus (Burge and Pfefferkorn, 1966a), influenza virus (Simpson and Hirst, 1968) and reovirus (Fields and Joklik, 1969). The results of chemical mutagenesis of SFV are shown in Table 3-3. The use of these 3 mutagens, each with a different mode of action, should enhance the chance of isolating mutants in several different genes. Out of many presumptive ts mutants isolated, 38 stable or fairly stable mutants (including those isolated by Sambrook) were selected for study. These mutants were numbered from ts-1 to ts-38.

Leak yield and reversion of mutants

ts mutants often undergo limited growth at the restrictive temperature (leak growth) and single-step mutants tend to revert to the parental or wild-type character resulting in an accumulation of wild-type virus in the mutant stocks. This wild-type content varies from stock to stock depending at which stage reversion arose; stocks will contain more wild-type virus if reversion occurred early in the growth cycle. Stocks with high leak or reversion rates are unsuitable for physiological and genetical studies and must be discarded.

Table 3-4. Some properties of SFV ts mutant

Mutagen[a]	ts	Plaque[b] size	Leak[c] yield	e.o.p.[d] at 38.3°	Mutagen	ts	Plaque size	Leak yield	e.o.p. at 38.3°
5-Fu	1	s	1.1×10^{-3}	$<1\times10^{-6}$	5-Fu	20	wt	4.1×10^{-3}	3×10^{-4}
5-Fu	2	s	2.2×10^{-4}	$<1\times10^{-6}$	HA	21	s	3.31×10^{-3}	$<1\times10^{-6}$
5-Fu	3	wt	5.8×10^{-4}	4.0×10^{-6}	HA	22	s	1.1×10^{-2}	1×10^{-3}
5-Fu	4	s	2.5×10^{-4}	5.0×10^{-6}	NTG	23	wt	1.3×10^{-4}	$<1\times10^{-6}$
5-Fu	5	v.s.	2.1×10^{-4}	$<1\times10^{-6}$	NTG	24	L	1×10^{-2}	1.3×10^{-3}
5-Fu	6	s	2.6×10^{-3}	2.6×10^{-5}	NTG	25	wt	3.7×10^{-5}	9.1×10^{-5}
5-Fu	7	v.s.	1.9×10^{-4}	$<1\times10^{-6}$	NTG	26	wt	4.3×10^{-2}	6.5×10^{-4}
5-Fu	8	wt	3.2×10^{-6}	$<1\times10^{-6}$	NTG	27	wt	3.6×10^{-4}	8.5×10^{-4}
5-Fu	9	s	6.2×10^{-4}	1.1×10^{-5}	NTG	28	s	5.1×10^{-5}	3.9×10^{-5}
5-Fu	10	wt	4.5×10^{-4}	3.0×10^{-4}	NTG	29	wt	2.4×10^{-4}	1.7×10^{-3}
HA	11	s	2.5×10^{-5}	$<1\times10^{-6}$	NTG	30	wt	2.6×10^{-4}	$<1\times10^{-6}$
HA	12	s	5.4×10^{-4}	7.2×10^{-4}	NTG	31	wt	1.0×10^{-4}	8.5×10^{-6}
HA	13	s	8.9×10^{-4}	$<1\times10^{-6}$	NTG	32	wt	3.2×10^{-4}	$<1\times10^{-6}$
HA	14	s	6.3×10^{-4}	$<1\times10^{-6}$	HA	33	wt	5.6×10^{-3}	8.5×10^{-6}
HA	15	L	4.9×10^{-5}	$<1\times10^{-6}$	HA	34	L	8.3×10^{-3}	$<1\times10^{-6}$
HA	16	wt	4.5×10^{-5}	$<1\times10^{-6}$	NTG	35	s	2.3×10^{-5}	$<1\times10^{-6}$
HA	17	wt	2.9×10^{-3}	1.2×10^{-4}	NTG	36	wt	5.5×10^{-3}	$<1\times10^{-6}$
HA	18	L	4.1×10^{-5}	$<1\times10^{-6}$	NTG	37	s	1.5×10^{-3}	6.6×10^{-6}
HA	19	wt	3.4×10^{-4}	2.3×10^{-4}	NTG	38	s	1.1×10^{-5}	1.6×10^{-6}

a. 5-Fu = 5-Fluorouracil, HA = Hydroxylamine, NTG = Nitrosoguanidine

b. determined at 28°, wt = wild-type size, s = small, smaller than wt, v.s. = very small, L = large, larger than wt.

c. Leak yield = $\dfrac{\text{Yield of virus grown at 38.3°}}{\text{Yield of virus grown at 28°}}$

d. e.o.p. at 38.3° = $\dfrac{\text{Titer of virus stock assayed at 38.3°}}{\text{Titer of same stock assayed at 28°}}$

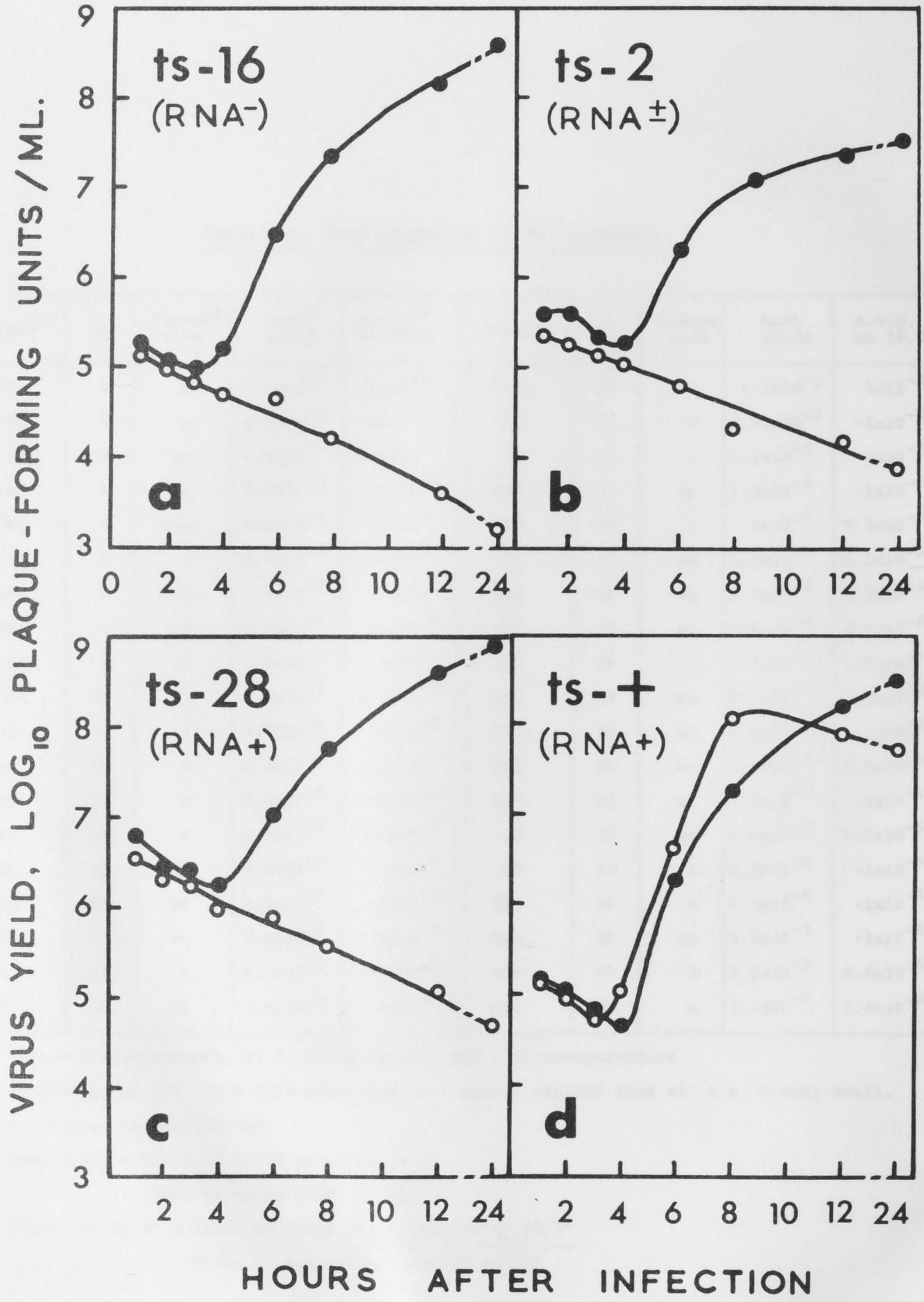


Figure 3-2. Growth curves of SFV and ts mutants. Infected minilayers were incubated at 30° (●——●) or 38.5° (o——o) and samples were removed at intervals for assay of infectivity. For an explanation of the RNA phenotype, see Chapter 4.

Leak yield at 38.3° and efficiency of plating (e.o.p.) at 38.3° (a measure of reversion rate) of the ts mutants were determined as described in Materials and Methods (Chapter 2). Most of the mutants have an acceptable leak rate and e.o.p. at 38.3° of 10^{-3} and 10^{-4} or less, respectively (Table 3-4).

Growth curves of wild-type virus and mutants at 30° and 38.5°

After overnight incubation at 4° with AMD, infected cells were washed and incubated at 30° or 38.5° in the presence of AMD. At 30° mutants and wild-type virus grew normally and a maximum yield was not obtained after 24 hours incubation (figure 3-2). The growth of wild-type virus at 38.5° was more rapid than at 30° and maximum yield was obtained 8 hours after infection. However, the final yield at 38.5° was less than that at 30°, probably due to AMD killing the cells faster at the higher temperature. As expected, mutants did not grow at 38.5°; the decreasing titer seen probably representing heat inactivation of uneclipsed virus (see Chapter 7).

Growth characteristics of mutants and wild-type virus at different temperatures

The growth of mutants at temperatures ranging from 28° to 38.3° was investigated to find the temperature at which inhibition of viral yield in a single growth cycle occurred. After a 2 hour adsorption period at room temperature, infected minilayers were washed and incubated at various temperatures in the absence or

Table 3-5. Yields of mutants grown at various temperatures in the absence and presence of AMD.

ts	AMD	24 hr yield (log_{10} PFU per ml) at 28°	32°	34°	36°	37°	38.3°
1	-	7.35	5.60	4.77*	3.00	2.47	<2.00
	+	7.65	6.69	5.81	4.30*	4.00	<2.00
4	-	7.60	6.84	7.04	5.60*	5.69	3.74
	+	7.60	6.95	7.36	6.30	6.47	4.84*
7	-	7.14	7.32	6.30	2.77*	2.77	<2.00
	+	7.79	7.60	6.60	6.95	6.84	4.14*
11	-	7.25	7.00	7.36	6.69	6.47	4.60*
	+	7.61	7.43	7.65	6.69	6.90	5.39*
12	-	7.57	6.60	5.69	5.77	6.69	4.30*
	+	7.47	6.84	6.30	6.17	5.30*	4.40
13	-	7.34	6.30	6.69	5.90	5.57	4.00*
	+	7.04	6.47	6.69	6.00	4.84*	4.90
15	-	6.90	7.38	7.60	6.47	4.69*	3.90
	+	7.30	7.69	8.07	6.60	6.47	5.14*
16	-	7.60	7.41	7.47	6.00	5.55*	2.60
	+	7.07	7.07	6.95	6.30	5.32	2.00*
18	-	7.23	6.60	5.69	4.30*	3.39	2.84
	+	7.20	7.23	6.00	4.90*	4.90	2.77
19	-	6.84	6.77	6.30	6.30	4.00*	2.60
	+	7.43	7.20	6.77	7.00	6.30	5.30*
21	-	7.17	6.69	6.30	5.47	5.39	4.69*
	+	7.49	7.23	6.00	5.54	5.20*	4.95
+	-	7.55	7.69	7.77	6.84	6.00	6.90
	+	7.47	7.34	7.62	7.60	7.63	7.43

Infected cells were incubated at various temperatures in the absence or presence of 0.1 µg AMD per ml. After 24 hr the viral yields were assayed at 28°.

* = temperature at which 99% or more inhibition of viral yield occurred.

presence of 0.1 µg AMD per ml (this infection procedure and the use of a low concentration of AMD was adopted by Sambrook (1965) and employed here to obtain comparable results). After 24 hours the yields obtained at the different temperatures were assayed at 28° and compared with the normal yield obtained at 28°.

In the absence of AMD, decreasing yields were obtained from 34° upwards (Table 3-5). For most mutants a 99 per cent inhibition (or 'cut-off') of normal yield occurred at 36° to 38°. Two mutants, ts-1 and ts-5, had a slightly lower cut-off temperature of 34°. In the presence of AMD the cut-off temperature may be increased up to 3° (e.g. ts-5, Table 10-1 in Chapter 10), due to an increase in yield at higher temperatures. AMD also increased the yields of most mutants at most temperatures of incubation. However, the cut-off temperature of ts-12, -13 and -21 was 1° lower in the presence of AMD than in its absence. This was due to an enhancement of the yield of ts-21 at lower temperature in the presence of AMD. The reason for the lower cut-off temperature of ts-12 and ts-13 in the presence of AMD is not known. A summary of the cut-off temperature of viral yield (incorporating Sambrook's data) is presented in Table 10-1 (see Chapter 10).

Ability of mutants to form plaques at different temperatures

The ability of the mutants to form plaques under agar was determined at various temperatures in the absence of AMD (AMD kills the cells before plaques can form as this is a multiple growth cycle event). The cut-off temperature of plaque-forming ability (Table 3-6)

Table 3-6. Ability of mutants to form plaques at various temperatures.

ts	Titer, $\log_{10}$ PFU at					ts	Titer, $\log_{10}$ PFU at				
	28°	32°	34°	36°	38.3°		28°	32°	34°	36°	38.3°
1	6.98	7.08	7.00	4.50*	<2.00	20	6.00	6.13	5.96	3.88*	2.37
2	7.53	7.74	7.32	4.51*	<2.00	21	8.33	8.18	8.30	7.77	<2.00*
3	7.28	7.27	6.34	6.82	1.88*	23	7.45	7.22	6.83	7.02	<2.00*
5	7.63	5.78	5.56*	<2.00	<2.00	26	7.79	7.74	7.74	7.20	4.63*
6	8.12	8.05	7.78	4.57*	3.54	27	6.40	6.59	6.21	5.48	2.62*
7	7.69	7.68	6.95	1.93*	<2.00	28	8.11	7.97	8.14	7.65	3.70*
8	7.60	7.53	7.64	6.85	<2.00*	29	7.11	7.10	7.11	7.06	4.22*
9	7.13	7.08	7.04	6.66	<2.00*	30	7.28	7.45	6.86	6.85	<2.00*
10	7.11	7.08	7.08	6.59	3.60*	31	7.11	7.04	6.57	6.54	2.04*
11	7.34	7.40	6.95	6.90	<2.00*	32	7.41	7.19	6.86	7.05	<2.00*
12	6.74	6.78	6.74	6.60	3.60*	33	7.45	7.40	7.04	7.18	2.38*
16	7.37	7.36	6.75	5.98	<2.00*	35	6.88	6.78	6.73	6.64	<2.00*
17	7.82	7.64	7.27	4.33*	3.81	37	7.29	7.13	6.61	3.95*	2.11
18	6.85	7.04	6.59	6.66	<2.00*	38	7.51	6.86	6.70	6.92	1.70*
19	7.28	7.04	7.15	6.75	2.23*	+	7.49	7.33	7.51	7.33	7.26

Cells were infected with dilutions of each mutant and incubated at various temperatures.

Plaques were counted after 3 days.

* = temperature at which 99% or more inhibition of plaque formation occurred.

(temperature at which 99 per cent inhibition of plaque formation occurred) is in most cases the same as the cut-off temperature of viral yield but may differ by about 2^{o} for some mutants.

Plaque size of mutants

Both wild-type virus and mutants produce both large and small plaques. These different sized plaques are probably not produced by variants as virus from such plaques will also give rise to plaques of different sizes. This phenomenon probably results from asynchrony of viral infection, the latent period in single growth cycles being quite variable, especially in primary cells.

The plaque size at 28^{o} of wild-type virus and mutants is indicated in Table 3-4. Very few mutants produce very large or minute plaques (figure 3-3). Both wild-type virus and mutants produce the largest plaques at about 32^{o}. At 38.3^{o} most mutants did not form plaques as expected but a few form 'leak' plaques - tiny plaques with indistinct outlines which are difficult to count. Such plaques were ignored in making plaque assays at 38.3^{o}.

Figure 3-3

TS-18
[large plaques]

WILD-TYPE
[medium plaques]

TS-38
[small plaques]

Plaques formed in chick embryo cell monolayers after 4 days incubation at 30°

(MAGNIFICATION x 1)

DISCUSSION

Semliki Forest virus offers a model system for the study of the replication of small animal riboviruses. The complete insensitivity of its replication to treatment with actinomycin D permits the execution of physiological, biochemical and genetical studies in essentially the total absence of host cell's macromolecular synthesis. The growth of SFV in the presence of AMD was quicker than in its absence. This probably resulted, in the presence of AMD, from the rapid take over of host cell synthetic mechanisms by the virus for the synthesis of viral component.

Conditional lethal mutants provide an elegant and powerful method for the elucidation of viral replication. This was first exploited by Epstein and his colleagues who, using the 2 classes of conditional lethal mutants - temperature-sensitive and amber mutants - have provided a fairly complete genetic map for the bacteriophage T4 and characterized the defects of the genes (Epstein et al, 1963). Only ts mutants are readily obtaining with animal viruses and studies varying in depth have been reported with viruses of several taxonomic groups (Fenner, 1969).

The universal nature of ts mutants provides a system for the total mapping and for the study of the physiology and biochemistry of animal viruses (Cooper, 1967a). To be of value, ts mutants (i) should be single-step mutants, i.e. defective (in respect of temperature-sensitivity) in one function only. Double or multiple-step mutants make interpretation of results

difficult. However, selected double mutants may be valuable in genetic experiments for mapping single-step mutants (Cooper, 1968), and (ii) must not be 'sisters' (i.e. the mutations must occur independently) and should, desirably, represent the entire viral genome.

ts mutants were readily recovered after treatment of SFV with 3 different mutagens to enhance the possibility of obtaining mutants in different genes. These mutants have different leak rates, e.o.p. at 38.3° and cut-off temperature of viral yield and plaque-forming ability and are therefore unlikely to be 'sisters'. Since the induced mutation rate (1-5 per cent) is low, there should be few double mutants (Cooper, 1967a). Thus the ts mutants of SFV should be suitable for both genetical and physiological studies.

CHAPTER 4

CHARACTERIZATION OF ts MUTANTS I :

VIRAL RNA SYNTHESIS

INTRODUCTION

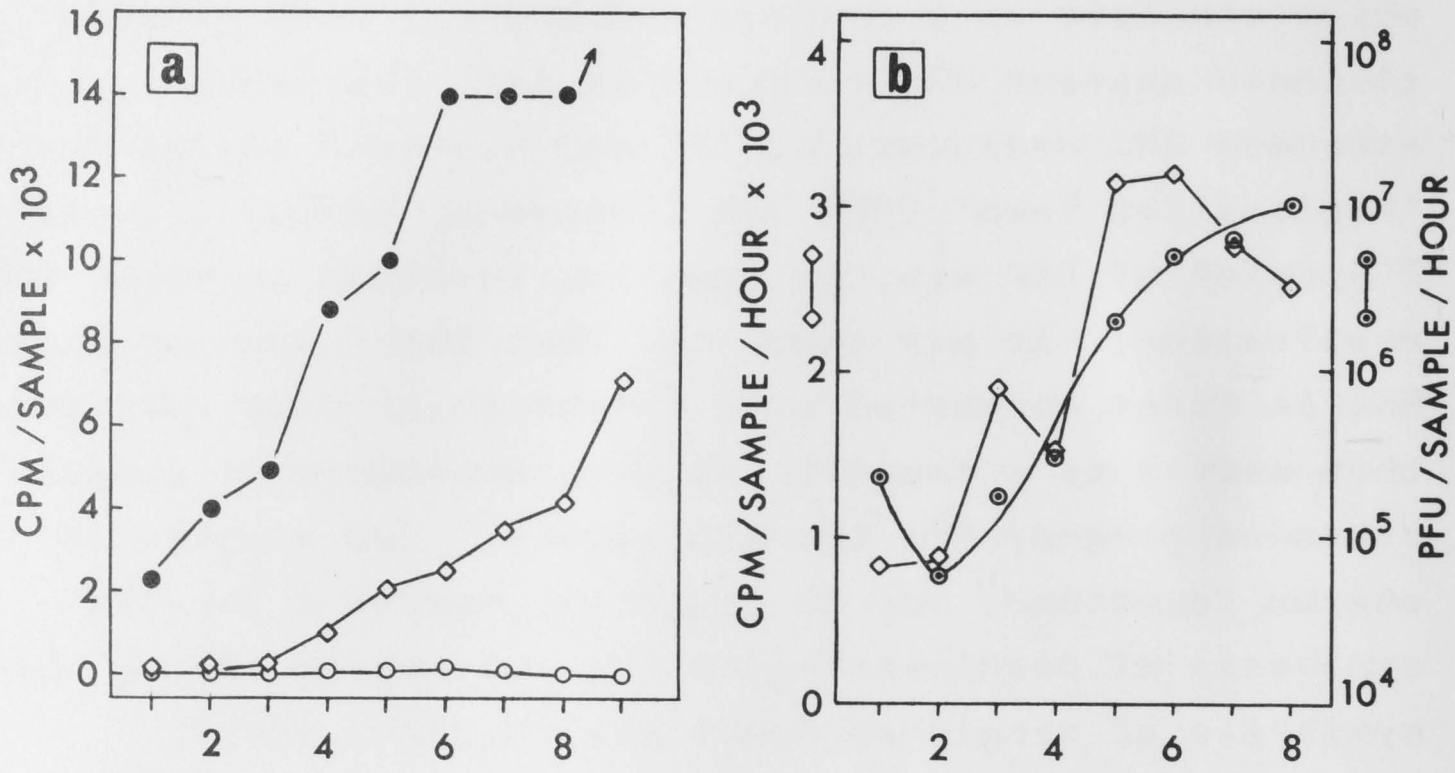
Semliki Forest virus ts mutants share the common characteristic of inability to form plaques at 38.3°. Their specific defects were investigated with physiological and biochemical tests. The first property of the mutants examined was their ability to synthesise virus-specific RNAs (which were later characterized) at the restrictive temperature. One group of mutants which were unable to synthesise virus-specific RNAs at the restrictive temperature was used to investigate the effects of viruses on cellular macromolecular biosynthesis in the absence of viral RNA synthesis.

Four forms of virus-specific RNAs have been extracted from the cytoplasm of cells infected with SFV. These RNA species sedimented in sucrose gradients at 42S, 26S, 20S and a polydisperse RNA from 14S-30S (Friedman et al, 1966; Mecs et al, 1967; Sonnabend et al, 1967; Friedman, 1968b). The 42S RNA was identified with the RNA found in purified virions. The 26S or 'Interjacent' RNA (Martin, 1966) is of unknown significance. Both 42S and 26S RNA are single-stranded and have the same base ratio and buoyant density in caesium sulphate. After heat denaturation, 42S RNA was found to sediment at 26S (Friedman et al, 1967). 42S RNA is infectious but 26S RNA possesses little, if any, infectivity. Various functions have been suggested for the 26S or interjacent RNA, such as that it is viral 42S RNA in a less compact form, viral messenger RNA, or, a break down product of 42S RNA (if 42S RNA was labile and broke in the middle into 2 strands) (Sonnabend et al, 1967;

Martin, 1966; Friedman et al, 1967). However, experimental evidence for any of these interpretations is lacking.

The polydisperse RNA sedimenting at 14S-30S was identified as the 'Replicative Intermediate' (RI) RNA which consists of a double-stranded RNA with single-stranded nascent RNA attached to it. The double-stranded RNA sediments at 20S and has been called the 'Replicative Form' (RF) RNA (Friedman, 1968b). These 2 species of RNA are, presumably, involved in viral RNA replication. It was suggested that the infecting viral RNA is first converted into a double-stranded form which then serves as a template for the synthesis of single-stranded progeny RNA (Martin, 1966). Two enzymes (or enzyme functions) may be required; enzyme I for the synthesis of double-stranded RNA and enzyme II for the synthesis of single-stranded RNA (Martin, 1966).

Figure 4-1



Synthesis of cellular and viral RNA.

(a) Cumulative RNA synthesis in chick cells. Minilayers were incubated in the presence of 5 µC/ml of ^{3}H uridine. Samples were removed at intervals and the acid insoluble radioactivity measured. ●—● = untreated cells, ○—○ = cells treated with 1 µg AMD/ml and ◇—◇ = cells treated with AMD and infected with SFV.

(b) Rate of synthesis of viral RNA and virus. At intervals the medium of minilayers infected in the presence of AMD was replaced with fresh medium containing AMD and 4 µC/ml of ^{3}H adenosine. One hour after the addition of ^{3}H adenosine, the growth medium was removed and assayed for extracellular virus. The cells were washed, lysed and the acid insoluble radioactivity was determined as described in materials and methods.

RESULTS

Cellular RNA synthesis

Actinomycin D (AMD) is known to inhibit cellular RNA synthesis by forming a complex with DNA (Reich and Goldberg, 1964). The effect of AMD on chick embryo cell RNA synthesis was investigated by incubating the cells in growth medium containing 1 µg/ml AMD and 5 µC/ml of ^{3}H uridine and measuring the incorporated radioactivity. AMD quantitatively inhibited cellular RNA synthesis (figure 4-1a). Untreated cells showed cumulative RNA synthesis.

A set of AMD-treated cells which was infected with SFV also showed cumulative RNA synthesis (figure 4-1a) but at a lower level than the uninfected cells not treated with AMD. This AMD-resistant RNA is viral in origin (shown below). Thus AMD-treated chick cells provide a satisfactory system for the study of viral RNA synthesis in the absence of cellular RNA synthesis.

Viral RNA synthesis

The rate of AMD-resistant RNA (or virus-specific RNA, shown later) synthesis was determined by labelling AMD-treated infected cells with ^{3}H adenosine for a 1 hour period at various times after infection (adenosine was used instead of uridine because it is incorporated into RNA more rapidly than uridine; E. M. Martin, personal communication). The rate of appearance of mature virus in the growth medium at intervals during the growth cycle was also determined (figure 4-1b).

Table 4-1. Ability of ts mutants to synthesise RNA

ts	C.P.M. at 38.3° I	II	III	% of wild-type RNA at 38.3° (average of I, II & III)	C.P.M. at 28°	% of wild-type RNA at 28°
C	110	77	88	-	86	-
+	3223	5623	2315	100	1336	100
1	84	62	81	2.0	890	66.6
2	214	184	114	4.6	237	17.8
3	90	91	118	2.7	336	25.1
4	624	589	148	12.2	1248	93.3
5	93	42	66	1.8	925	69.2
6	79	68	61	1.9	1294	96.8
7	79	87	65	2.1	811	60.7
8	917	1349	326	23.2	625	46.8
9	62	63	67	1.7	3090	231.3
10	75	63	78	1.9	185	13.8
11	74	62	75	1.9	1982	148.3
12	664	525	464	14.8	511	38.3
13	668	631	391	15.1	915	68.5
14	658	891	464	18.0	577	43.2
15	693	457	275	12.8	988	73.9
16	97	59	76	2.1	132	9.9
17	1503	2570	430	40.3	791	59.2
18	917	1175	582	24.0	169	12.6
19	96	76	65	2.1	429	32.1
20	1079	2692	894	41.9	1185	88.7
21	613	724	228	14.0	1272	95.2
22	1790	2138	1776	51.1	1582	118.4
23	2141	2291	340	42.8	466	34.9
24	1834	2512	934	47.3	1073	80.3
25	2915	3631	769	65.5	1169	87.5
26	2907	3802	905	68.2	926	69.3
27	3840	3981	389	73.6	768	57.5
28	3958	3467	1424	79.3	132	9.9
29	3530	3467	802	69.6	1120	83.8
30	3243	3311	812	66.0	998	74.7
31	4522	4169	1154	88.2	1626	121.7
32	3165	3631	1062	70.4	910	68.1
33	2711	3236	833	60.7	927	69.4
34	2089	2138	322	40.8	407	30.5

Minilayers infected in the presence of AMD with 5-10 PFU per cell were incubated at 28° or 38.3°. 2 hr after infection the medium was replaced with fresh medium containing AMD and ^{3}H uridine (2 μC/ml). 6.5 hr after infection ^{3}H uridine incorporated into RNA was measured as described in materials and methods. All the mutants were tested 3 times at 38.3° and once at 28°. All tests were done in duplicate. Wild-type virus is denoted by "+" and AMD-treated uninfected cells by "C".

Maximum viral RNA synthesis occurred at about 6 hours after infection with a subsidiary peak of synthesis at 3 hours. Similar results were obtained by Taylor (1965) and Mecs et al (1967). The significance of the early (3 hour) peak of RNA synthesis is not known. The rate of production of extracellular virus was slower and maximum yield had not been reached by 8 hours after infection.

On the basis of the above experiment, subsequent analysis of viral RNA synthesis at 38^{o} (or thereabouts) was made at 6.5 hours after infection.

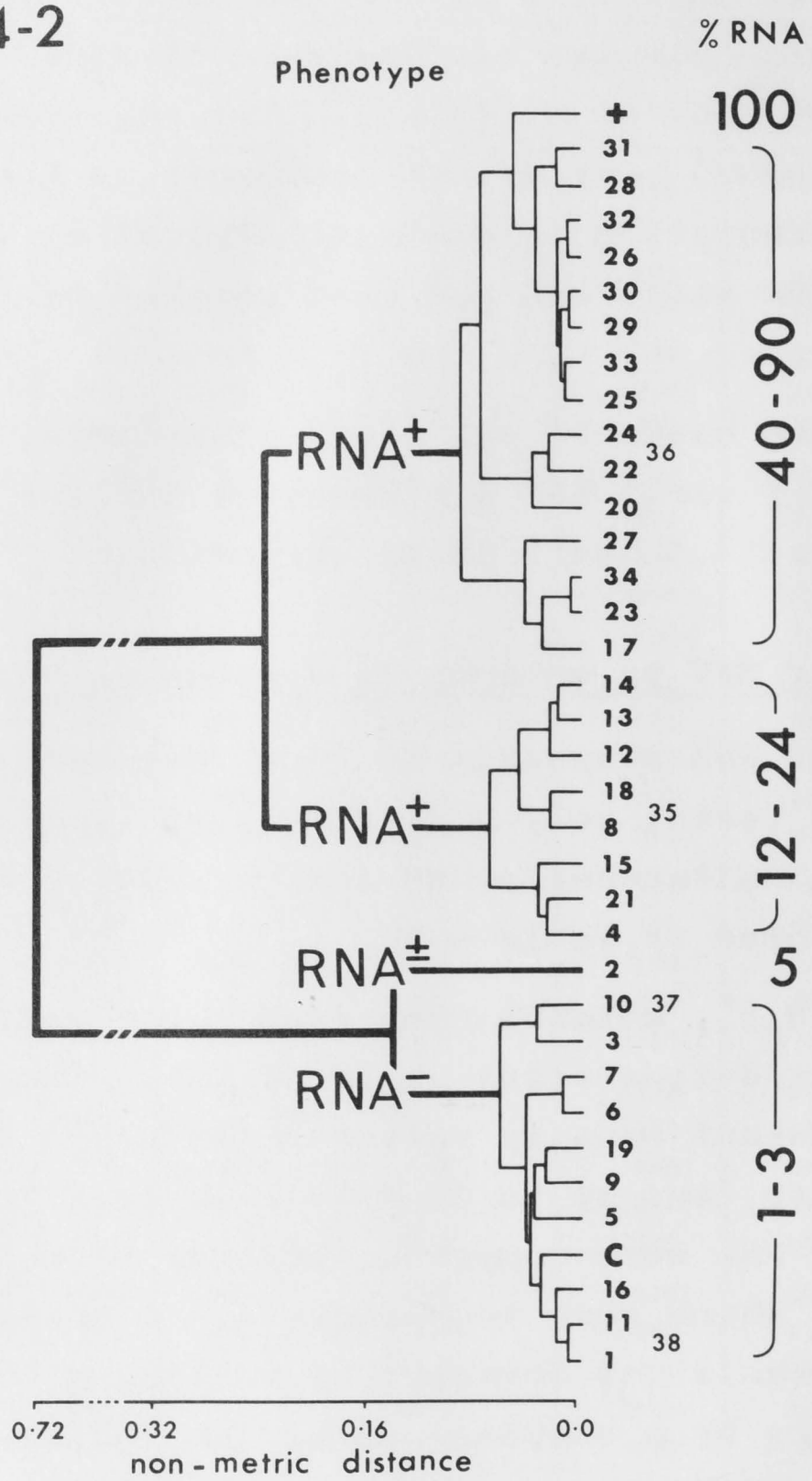
Ability of SFV ts mutants to synthesise RNA

Viral RNA synthesis at 38.3^{o} was measured in 5 replicate tests; only 3 of the tests involved all the mutants simultaneously and the results of these tests are presented in Table 4-1.

At 38.3^{o}, mutants made from 1 per cent to 90 per cent of wild-type virus yield of RNA. The total RNA made by each mutant usually varied less than 4 fold between experiments (but up to 10 fold with ts-27). When their yields of RNA were compared, mutants were found to cluster in groups which were reproducible. A consistent grouping of the mutants was obtained by analysing the data from the 5 tests by a computer using the 'MULTCLAS' program of Lance and Williams (1967).

The computer was programmed to calculate the differences in the amounts of RNA made at 38.3^{o} between all possible pairs of mutants. The relative differences between the mutants were expressed as relative distances

Figure 4-2



Grouping of SFV ts mutants according to their ability to synthesise RNA at 38.3°. The amount of RNA synthesised by mutants of each group is shown as a percentage of wild-type on the right, and the coefficient at which the groups fromed (a measure of group heterogeneity) on the lower scale. ts 35, 36, 37 and 38 are new isolates and were not included in the computer grouping. Wild-type virus is denoted by "+" and AMD-treated uninfected cells by "C".

(non-metric coefficient; Lance and Williams, 1966) for the construction of a dendogram. At the beginning of the construction of the dendogram, the pair of mutants showing the least relative distance was sorted out to form a group (which we shall call '*ts*-X') which replaces the two mutants in subsequent calculations. The distances between the mutants in this new matrix (*ts*-X and the remaining mutants) were calculated. The pair of mutants showing the least distance in this matrix was sorted out to form another new group which replaces the 2 mutants in subsequent calculations. This procedure of sorting out pairs of mutants was repeated till eventually a single group was formed from all the original mutants. The computer was also programmed to print out the dendogram on a chart recorder and this result was redrawn in figure 4-2. The mutants were separated by arbitrary equal spacings in one axis (vertical) and the successive fusions of the mutants or groups at the indicated levels were recorded in the horizontal axis.

This computer grouping of the mutants is not a 'true' classification. It is an arbitrary grouping which can be used to facilitate the selection of particular mutants for further characterization.

Grouping of SFV *ts* mutants based on their ability to synthesise RNA at 38.3°

Most of the mutants were placed into 2 groups by the computer. One mutant, *ts*-2, which was different from the above 2 groups in most tests, was placed in a group of its own. From a consideration of the amount of RNA synthesised and the physiological behaviour of the

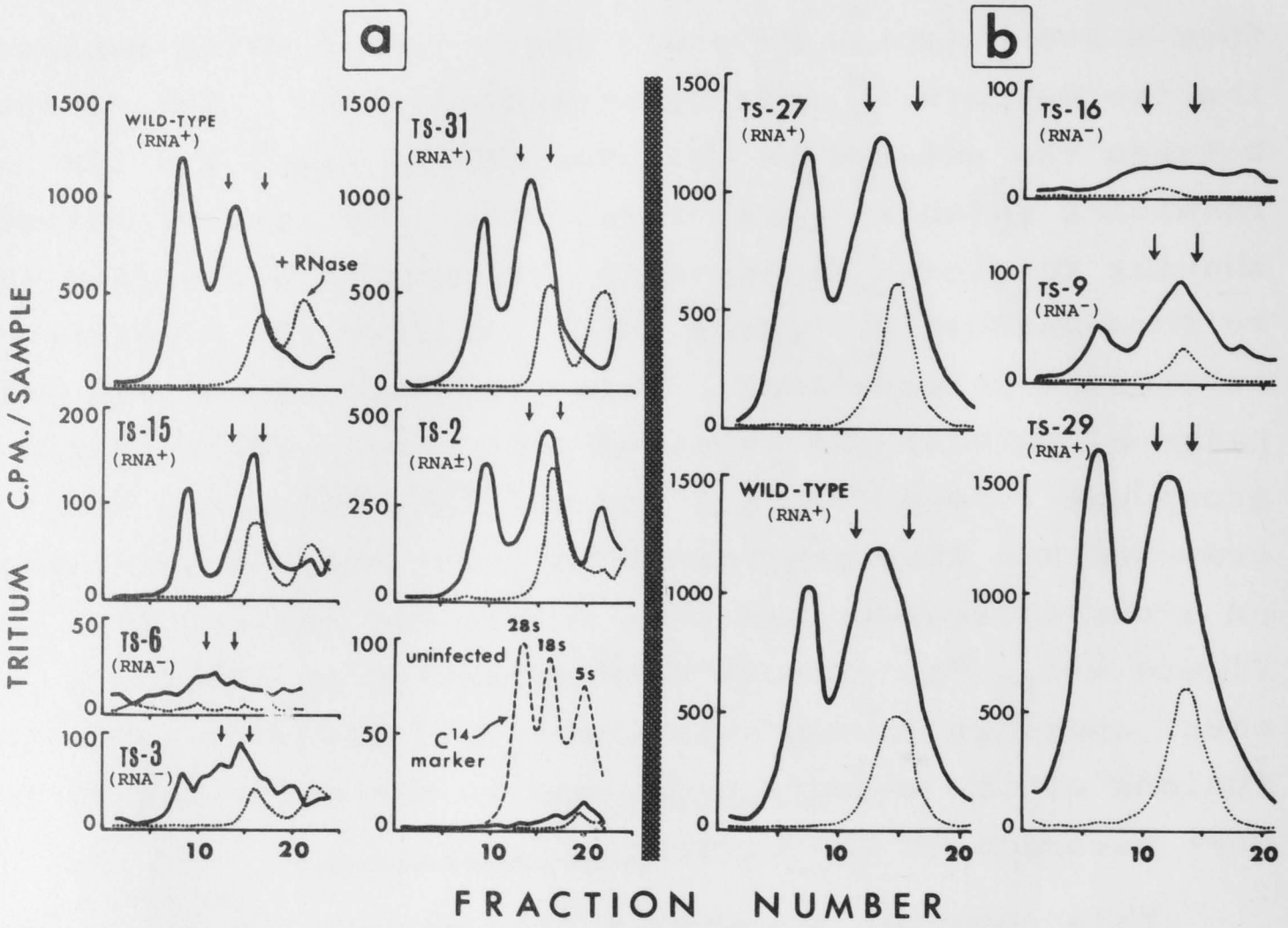


Figure 4-3. SUCROSE GRADIENT ANALYSIS OF VIRAL RNA. CELLS INFECTED AT 38.3° IN THE PRESENCE OF AMD WERE LABELLED WITH ^{3}H URIDINE FROM 2-6.5 HR AFTER INFECTION. RNA WAS EXTRACTED FROM THE CYTOPLASM OF THE CELLS WITH SDS/PHENOL, ALCOHOL PRECIPITATED AND DISSOLVED IN A SMALL VOLUME OF BUFFER. THE RNA WAS SEDIMENTED IN A 6-30% SUCROSE GRADIENT IN A SW39 ROTOR FOR 2.5 HR AT 38,000 R.P.M. CHICK RIBOSOMAL RNA LABELLED WITH ^{14}C URIDINE WAS SEDIMENTED IN A SEPARATE GRADIENT TO ACT AS A MARKER AND THE POSITIONS OF 28S AND 18S RNA ARE INDICATED BY ARROWS. ACID INSOLUBLE RADIOACTIVITY WAS MEASURED AS DESCRIBED IN MATERIALS AND METHODS (——— = TOTAL COUNTS, ------- = + RNase COUNTS).

[a] HALF THE SAMPLE WAS TREATED WITH 1μg/ML OF RIBONUCLEASE FOR 10 MIN AT 4° BEFORE CENTRIFUGATION. (NOTE: TS 15 SYNTHESISED ABNORMALLY LOW LEVELS OF RNA IN THIS EXPERIMENT)

[b] AFTER CENTRIFUGATION, HALF OF EACH GRADIENT FRACTION WAS TREATED WITH RIBONUCLEASE.

mutants (see Chapters 4 to 7) at 38.3°, the 3 groups were designated RNA^-, $RNA^{\pm}$ and RNA^+ (figure 4-2).

The temperature-sensitivity of RNA synthesis of the 3 groups of mutants was not related to the mutants' leak growth or e.o.p. at 38.3°, or to their cut-off temperatures of yield and of plaque-forming ability (Bellett and Tan, unpublished observations).

At 28° some of the mutants seemed to be defective in RNA synthesis when compared with wild-type virus (Table 4-1). The results of this test are not meaningful because (i) the test was done only once, and (ii) the RNA made during the first 6.5 hours of infection was measured assuming that wild-type virus and the mutants multiply at the same rate; this is probably not so (see Chapter 3).

Sucrose gradient analysis of virus-specific RNAs

Virus-specific RNAs labelled with ^3H uridine were extracted from the cytoplasm of infected cells and analysed on 6-30 per cent sucrose gradients. RNA extracted from cells infected with wild-type virus sedimented mainly at 42S and at 22S (figure 4-3a). The radioactivity at the top of the gradient probably represents small pieces of degraded RNA. When the RNA preparation was treated with ribonuclease before centrifugation, only one RNA species remained, which sedimented at 20S (peak at top of gradient is degraded RNA). The 20S RNA can also be detected by treating each gradient fraction, collected after centrifugation, with ribonuclease (figure 4-3b). This suggested that

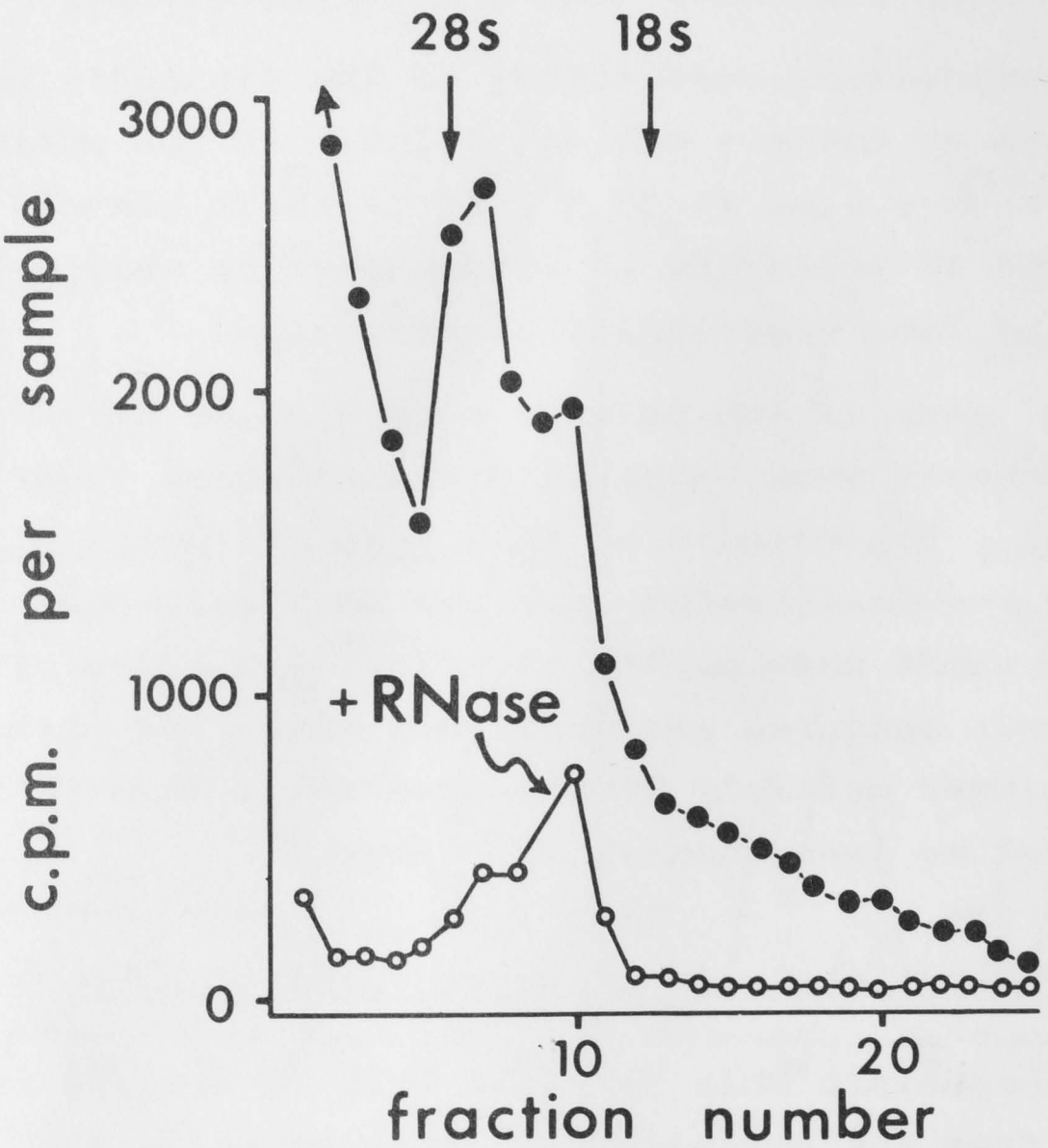


Figure 4-4. A cytoplasmic extract prepared by the same methods as in figure 4-3 was centrifuged for 16 hr at 24,000 r.p.m. in a SW25 rotor. One ml fractions were collected. Half of each fraction was treated with 5 μg per ml of ribonuclease for 30 min at room temperature. Acid-insoluble radioactivity was measured as described.

the 22S peak contained more than one species of RNA. Indeed, with prolonged centrifugation the 22S peak can be resolved into a 26S and a 20S RNA. Only the 20S RNA was resistant to ribonuclease treatment (figure 4-4).

The 42S, 26S and 20S RNA are the viral, interjacent and double-stranded or replicative-form RNA, respectively. No attempt was made to identify the replicative intermediate RNA.

Virus specific RNAs synthesised by 6 RNA^- mutants (ts-3, -6, -7, -9, -11, -16, -37), the $RNA^{\pm}$ mutant ts-2, and 10 RNA^+ mutants (ts-15, -17, -23, -27, -29, -30, -31, -32, -33, -35) were examined on sucrose gradients and 2 experiments are presented in figure 4-3. The wild-type virus gradient profile was variable (this was also observed by E. M. Martin; personal communication), probably due to differences between different batches of chick cells.

The control AMD-treated uninfected cells contained almost no labelled RNA and most RNA^- mutants behaved like ts-6 (shown in figure 4-3a) in failing to synthesise virus-specific RNAs. However, ts-3 (figure 4-3a) and ts-9 (figure 4-3b) made a slight but detectable amount of ribonuclease-resistant RNA.

All the RNA^+ mutants and the $RNA^{\pm}$ mutant ts-2, made all 3 species of virus-specific RNAs but in different proportions to those of wild-type virus. The RNAs of wild-type virus and of the mutants in the experiment shown in figure 4-3a were compared quantitatively. In this experiment the wild-type virus gradient profile was 'normal', i.e. similar to those reported by Friedman et al (1966) and Sonnabend et al (1967). The results

TABLE 4-2. ANALYSIS OF VIRUS-SPECIFIC RNAs

TS MUTANT	RNA PHENOTYPE	% OF TOTAL RNA*		
		42S	22S RNA	
			RNASE-RESISTANT	INTERJACENT
2	±	37	33	30
15	+	33	29	38
31	+	35	20	45
WILD-TYPE	+	44	15	41

* The amount of RNA was estimated by cutting out and weighing the area under each peak in figure 4-3.

are presented in Table 4-2. The most striking difference between the mutant and wild-type virus RNAs is seen in the proportion of ribonuclease-resistant RNA. Mutants synthesising low levels of RNA at 38.3° (e.g. ts-2) made proportionally more ribonuclease-resistant RNA than mutants synthesising a wild-type yield of RNA (e.g. ts-31), or wild-type virus. In ts-2, ribonuclease-resistant RNA constituted about 33 per cent of the total RNA made, compared with 15 per cent in wild-type virus. Thus ts-2 and a number of other RNA^+ mutants (e.g. ts-15, -17, -30, -32, -33) seemed to be defective in the synthesis of single-stranded RNA.

Inhibition of cellular RNA synthesis by RNA^- mutants

SFV does not inhibit cellular RNA synthesis until late in infection (Taylor, 1965). RNA^- mutants were used to test if inhibition of cellular RNA synthesis (measured by ^3H uridine incorporation) could occur in the absence of viral RNA synthesis. Virus stocks were dialysed against growth medium to remove traces of AMD (virus stocks were prepared in the presence of 0.1 µg AMD per ml) before use. Adsorption of virus to cells was at 4° for 1 hour.

There was no significant difference at the 5 per cent level (F test) in the amount of radioactivity incorporated by uninfected cells and by cells infected with RNA^- mutants or with ts-2 ($RNA^{\pm}$) at 38.3° (Table 4-3). However, if cells infected with RNA^- mutants were preincubated for 1 hour at 28° (to allow some viral replication) and then transferred to 38.3°, inhibition of cellular RNA synthesis could readily be demonstrated

TABLE 4-3

INHIBITION OF CELLULAR RNA SYNTHESIS BY TS MUTANTS

TS MUTANT	^{3}H URIDINE CPM PER MINILAYER	
	6 HR AT 38.3°	1 HR AT 28° THEN 5 HR AT 38.3°
1 (RNA$^-$)	5420	1000
3 (RNA$^-$)	6271	3165
6 (RNA$^-$)	5107	1019
7 (RNA$^-$)	5358	3039
9 (RNA$^-$)	6071	3544
10 (RNA$^-$)	4574	3377
16 (RNA$^-$)	4801	1893
19 (RNA$^-$)	5632	954
2 (RNA$^\pm$)	5032	2484
23 (RNA$^+$)	ND	5013
30 (RNA$^+$)	ND	5439
WILD-TYPE	ND	5535
UNINFECTED	5413	5333

Cells were labeled with 4 μC of ^{3}H uridine per ml from the fourth to the sixth hour of incubation in growth medium lacking AMD and the total acid-insoluble radioactivity was determined as described in materials and methods.

ND = not determined.

(counts of radioactivity in uninfected and in infected cells were significantly different at the 0.1 per cent level, F test). Under these conditions the viral yield did not exceed 6 times that produced by cells incubated throughout at 38.3°.

In cells infected with wild-type virus or RNA^+ mutants, there was apparently no inhibition of cellular RNA synthesis. However, since substantial amounts of viral RNA are synthesised late in infection (figure 4-1a), inhibition of cellular RNA synthesis must have occurred otherwise infected cells would have incorporated more radioactivity than uninfected cells. Similar observations were reported by Taylor (1965).

DISCUSSION

The inability of RNA^- mutants to synthesise RNA at the restrictive temperature could have a number of causes. Firstly, there may be a defect in the synthesis of the enzyme RNA polymerase which is required for viral RNA synthesis. Evidence for this is presented in Chapter 6. Martin (1969) studying the polymerase of 3 SFV RNA^- mutants (ts-3, -5, -6), arrived at a similar conclusion. Secondly, the polymerase is produced but its activity may be unstable at the restrictive temperature. But, we have no evidence for this.

The $RNA^{\pm}$ mutant, ts-2 and RNA^+ mutants (e.g. ts-15) which made significantly less RNA than wild-type virus at 38.3^o, are characterized by the relatively high proportion of double-stranded (20S) RNA synthesised. The simplest explanation for the defect of these mutants is a restricted production of RNA polymerase II (if 2 enzymes are required for viral RNA synthesis); or, the production of a temperature-sensitive polymerase II whose activity is unstable at 38.3^o, resulting in reduced production of single-stranded RNA from the double-stranded template. But we have no evidence for this.

In infected cells there is an intimate relation between viral RNA and protein synthesis. Viral proteins other than polymerase may have a function essential for RNA synthesis. For example, a protein may be required to remove nascent RNA from the double-stranded template (Martin, 1966). Failure to do so may result in the

blocking up of the template and hence of single-stranded RNA synthesis. We have not tested this possibility with SFV ts mutants.

RNA^+ mutants made up to 90 per cent of wild-type virus yield of RNA at 38.3°, but produced less than 0.6 per cent of the wild-type yield of infectious virus under similar conditions. These mutants must be defective in functions required for virion formation which take place subsequent to RNA synthesis. These functions involve the synthesis and maturation of viral structural proteins (see next Chapter).

Disappointingly, the analysis of RNA made by RNA^+ mutants did not give any indication of the role of interjacent RNA in viral RNA replication. RNA^- mutants, as expected, did not make any RNA detectable in sucrose gradients except for ts-3 and ts-9. The significance of the RNA made is not known. It could be due to 'leak' synthesis. It is also possible that the input RNA was converted to a double-stranded form and polymerase II was lacking to continue with the RNA replication cycle. Analysis of the RNA of these 2 mutants in temperature shift-up experiments (see Chapter 6) should clarify the situation.

We have confirmed an earlier report that SFV inhibits host cell RNA synthesis late in the growth cycle of the virus (Taylor, 1965). Inhibition of cellular RNA synthesis was more satisfactorily demonstrated with RNA^- mutants. However, it seems that RNA^- mutants inhibited host RNA synthesis only if prior viral replication had occurred. This observation and the fact that wild-type virus inhibited host RNA

synthesis late in infection, suggest that progeny viral RNA exerted this effect; a situation clearly different from poliovirus where the inhibiting effects on host protein and RNA synthesis are apparently mediated by input viral RNA (Bablanian et al, 1965).

CHAPTER 5

CHARACTERIZATION OF *ts* MUTANTS II : VIRAL PROTEIN SYNTHESIS

INTRODUCTION

Subsequent to viral RNA synthesis in SFV infected cells, viral structural proteins are produced; nucleocapsids in the cytoplasm and viral membrane protein in cellular membranes (Acheson and Tamm, 1967; Friedman, 1968d). Nucleocapsids acquire an envelope by budding at the modified cell membrane and are released as mature virions. The ability of RNA^+ mutants to produce viral structural proteins was investigated.

Polyacrylamide gel electrophoresis of proteins extracted from cells infected with SFV revealed the presence of 6 virus induced proteins (Friedman, 1968d; Hay et al, 1968). Three of these proteins were identified as viral structural proteins; one located in the envelope (membrane protein) and two in the nucleocapsid (Friedman, 1968d). The synthesis of these proteins preceded the synthesis of virions (Sreevalsan and Allen, 1968) as would be expected since they are precursors of the virions.

In the electron microscope, nucleocapsids have been observed in the cytoplasm of SFV infected cells (Acheson and Tamm, 1967; Erlandson et al, 1967). SFV nucleocapsids extracted from infected cells and from purified virions have identical properties, namely:

(i) they have a lower sedimentation coefficient but a higher specific gravity (140-145S and 1.27-1.29 gm/ml, respectively, in potassium tartrate) than virions (300-350S and 1.17-1.18 gm/ml respectively);

(ii) they contain only 2 proteins; there are 3 in the virions;

(iii) they are unable to form plaques but contain infectious 42S RNA;

(iv) they are resistant to ribonuclease treatment and are not dispersed by EDTA so that they are not artefacts due to aggregates of ribosomes; and

(v) they probably have cubic symmetry (Acheson, 1968; Acheson and Tamm, 1967; Friedman and Berezesky, 1967).

The site of synthesis of viral polypeptides is not known but completed nucleocapsids are frequently associated with membrane structures or lining the cell membrane.

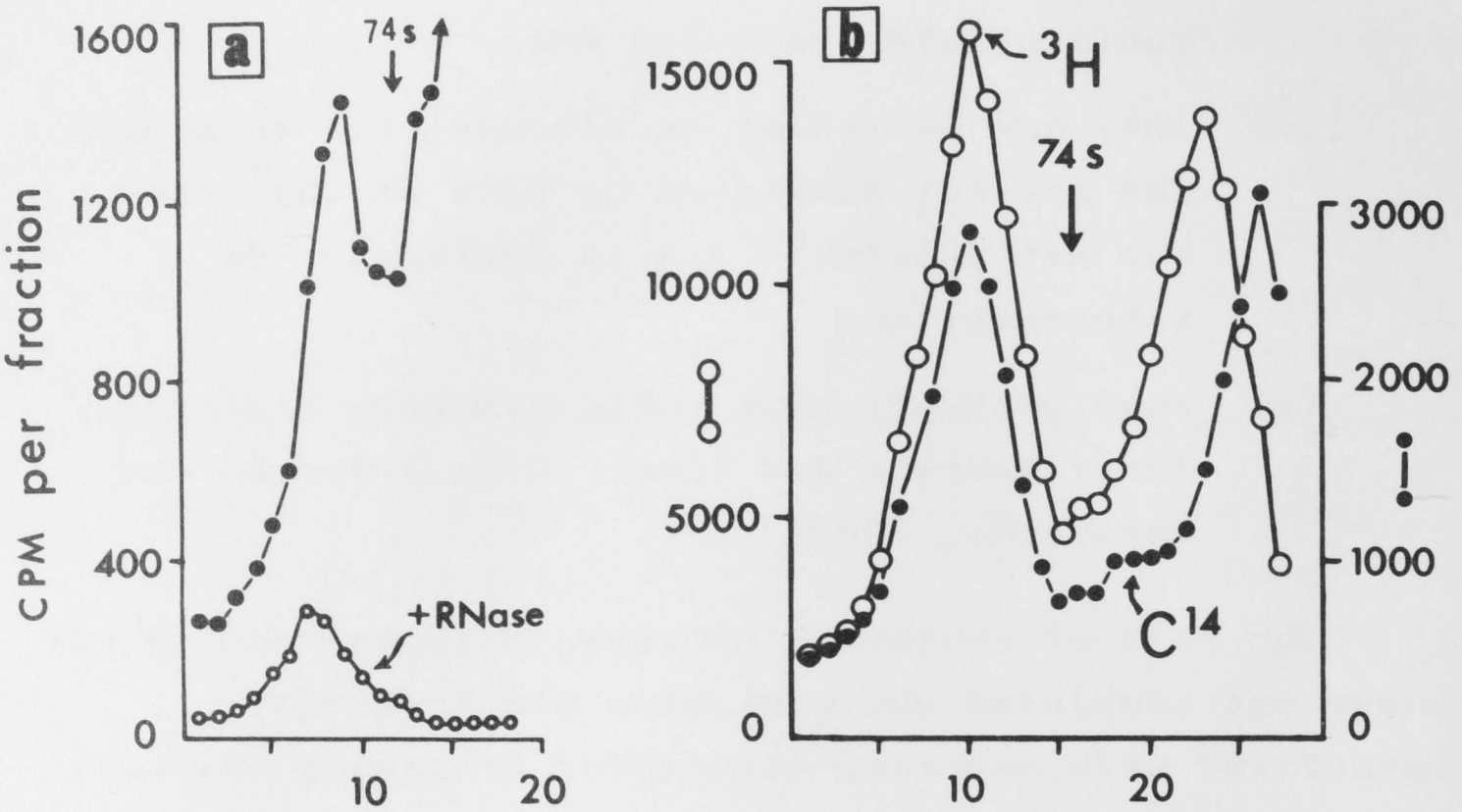


Figure 5-1. Sucrose gradient analysis of SFV nucleocapsids. 8 hr after infection cells were disrupted and centrifuged at 800 x g for 10 min. The pellet containing less than 20% of the total radioactivity was discarded. The supernatant was sedimented on a 15-30% sucrose gradient for 60 min at 38,000 r.p.m. in a SW 39 rotor. Purified chick liver ribosomes were sedimented on a separate gradient to serve as a 74S optical density marker. Sedimentation is from right to left.

(a) Infected cells were labeled with 3H uridine. Half the sample was treated with 1 µg per ml ribonuclease for 25' at room temperature before centrifugation.

(b) Analysis of cytoplasmic extract of infected cells doubly labeled with 3H uridine and C^{14} reconstituted protein hydrolysate.

RESULTS

The nucleocapsid of wild-type virus

The properties of nucleocapsids extracted from the cytoplasm of infected cells were examined in a series of experiments.

(1) Resistance to ribonuclease treatment

Cells infected in the presence of AMD at 38° were labelled with ^{3}H uridine from 2-8 hours after infection. A cytoplasmic extract was prepared and divided into 2 portions. One sample was treated with 1 µg per ml of ribonuclease in 0.1 M NaCl for 25 minutes at room temperature. The samples were then centrifuged separately on 15-30 per cent sucrose gradients for 60 minutes at 38,000 rpm in a SW 39 rotor.

A peak of radioactivity was observed at about 140S and it was fairly sensitive to ribonuclease treatment (figure 5-1a). The 140S was not well resolved from the radioactivity (rendered acid soluble by ribonuclease digestion, free RNA?) present near the top of the gradient and the gradient profile was not reproducible. Addition of Nonidet P40 (NP40, suggested by E. M. Martin, personal communication) and ribonuclease to final concentrations of 0.1 per cent and 0.1 µg per ml, respectively, to the cytoplasmic extract just before sedimentation resulted in good resolution of the 140S material (e.g. figure 5-1b) which was reproducible. NP40 and ribonuclease were incorporated in the extraction procedure in all subsequent experiments.

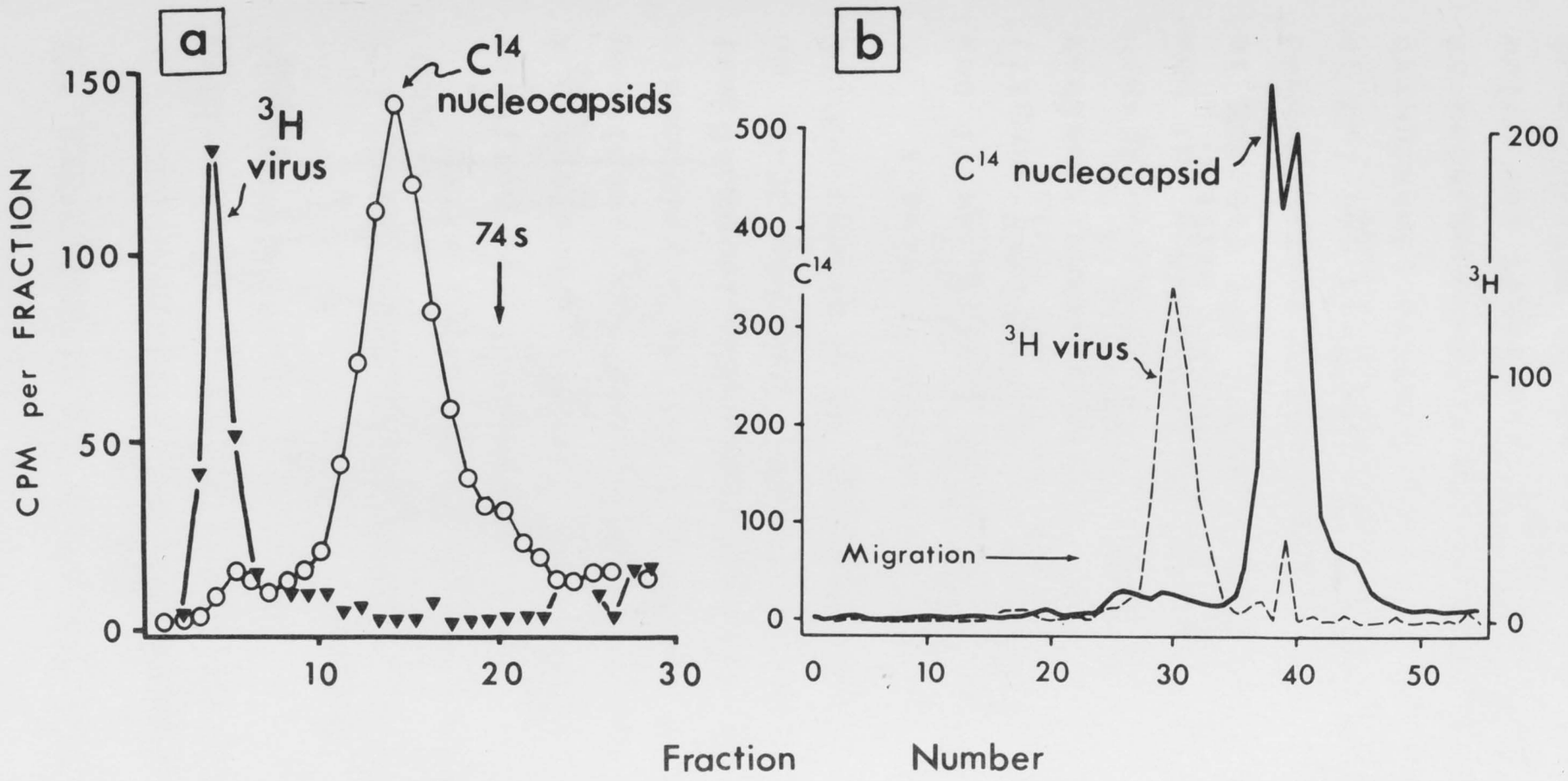


Figure 5-2. Identification of nucleocapsids.

(a) The 140S fraction from a sucrose gradient analysis of cytoplasmic extract of infected cells labeled with C^{14} reconstituted protein hydrolysate was mixed with ^3H leucine labeled purified virus. The mixture was sedimented on a 15-30% sucrose gradient for 30 mins at 38,000 r.p.m. in a SW39 rotor. Sedimentation is from right to left.

(b) A mixture of C^{14} reconstituted protein hydrolysate labeled 140S material from infected cells and ^3H leucine labeled purified virus was solubilized with SDS and subjected to polyacrylamide gel (7.5%) electrophoresis by a modified method of Summers et al., 1965.

(2) Nucleocapsids contain both RNA and protein

A cytoplasmic extract of infected cells doubly labelled with ^{3}H uridine and C^{14} reconstituted protein hydrolysate was analysed in a sucrose gradient. Both ^{3}H and C^{14} radioactivities (the settings on the channels of the scintillation counter were such that negligible ^{3}H counts appeared in the C^{14} channel, and corrections were made for C^{14} counts appearing in the ^{3}H channel) were present in the 140S peak, showing that this material contained both RNA and protein (figure 5-1b).

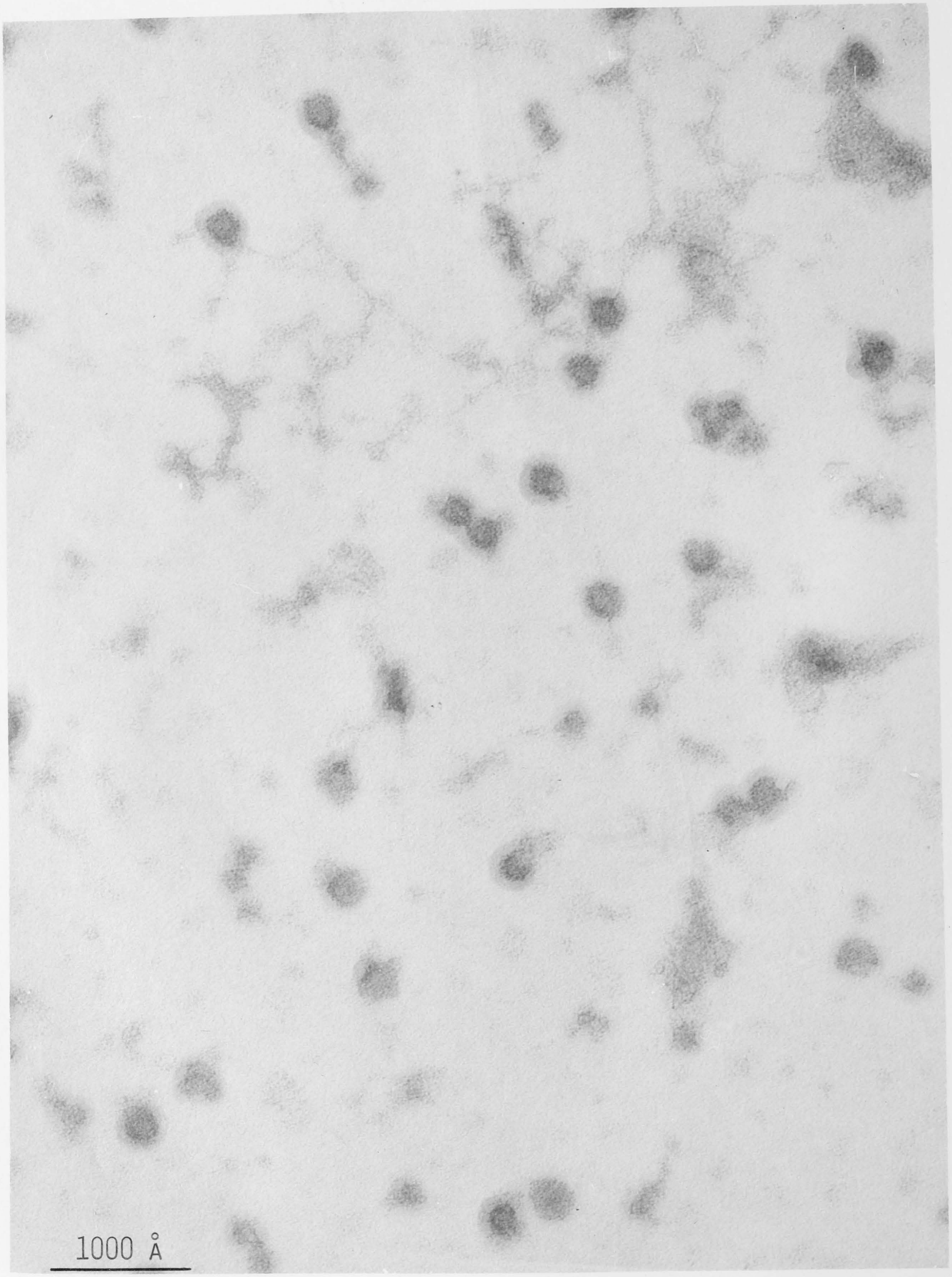
(3) Sedimentation of nucleocapsids and virions

The 140S material (labelled with C^{14}) from a sucrose gradient and ^{3}H labelled virions purified in a caesium chloride gradient were separately dialysed against buffer (0.01 M KCl, 0.00015 M $MgSO_4$, 0.01 M Tris pH 7.4) to remove sucrose and caesium chloride. The dialysed samples were mixed and analysed on a 15-30 per cent sucrose gradient (figure 5-2a). Under the same conditions, virions sedimented at about 300-350S.

(4) Polyacrylamide gel electrophoresis of nucleocapsid protein

The 140S material (labelled with C^{14} reconstituted protein hydrolysate) from a sucrose gradient was mixed with purified virions labelled with ^{3}H leucine and the mixture was solubilised with SDS and electrophoresed in a polyacrylamide gel (figure 5-2b). The 140S material contained a single major protein which migrated in exactly the same position as the faster moving protein found in purified virions (previously identified as the nucleocapsid protein; C. Burrell, personal communication).

PLATE 5-1



(x 247,000)

The split in the peak of the nucleocapsid protein in figure 5-2b is an artefact. In other experiments only one peak was obtained. A minor nucleocapsid protein may be present in fraction 26. This was not resolved in the 7.5 per cent gel used but can be resolved in a 10 per cent gel (C. Burrell, personal communication).

(5) Morphology and size of nucleocapsids

A sample of 140S material from a sucrose gradient was applied onto a formvar-coated grid, stained with sodium silicotungstate and examined in an electron microscope. Particles measuring about 230A in diameter were seen (Plate 5-1). These lack an envelope characteristic of and are smaller than virions. The particles are similar in size and in shape to nucleocapsids observed in thin sections of infected cells (see Chapter 8).

(6) Attempts to produce plaques with the 140S material or to demonstrate the presence of 42S RNA in them were unsuccessful. This was later found to be due to the extraction technique (see discussion below).

The above experiments demonstrate that the 140S material is the nucleocapsid of SFV virions and will be referred to as such in the following text.

Ability of mutants to synthesise nucleocapsids at 38.5°

The ability of the mutants to form nucleocapsids at 38.5° was tested in 2 experiments, shown in firures 5-3 and 5-4. For controls, nucleocapsid formation at 30° was tested.

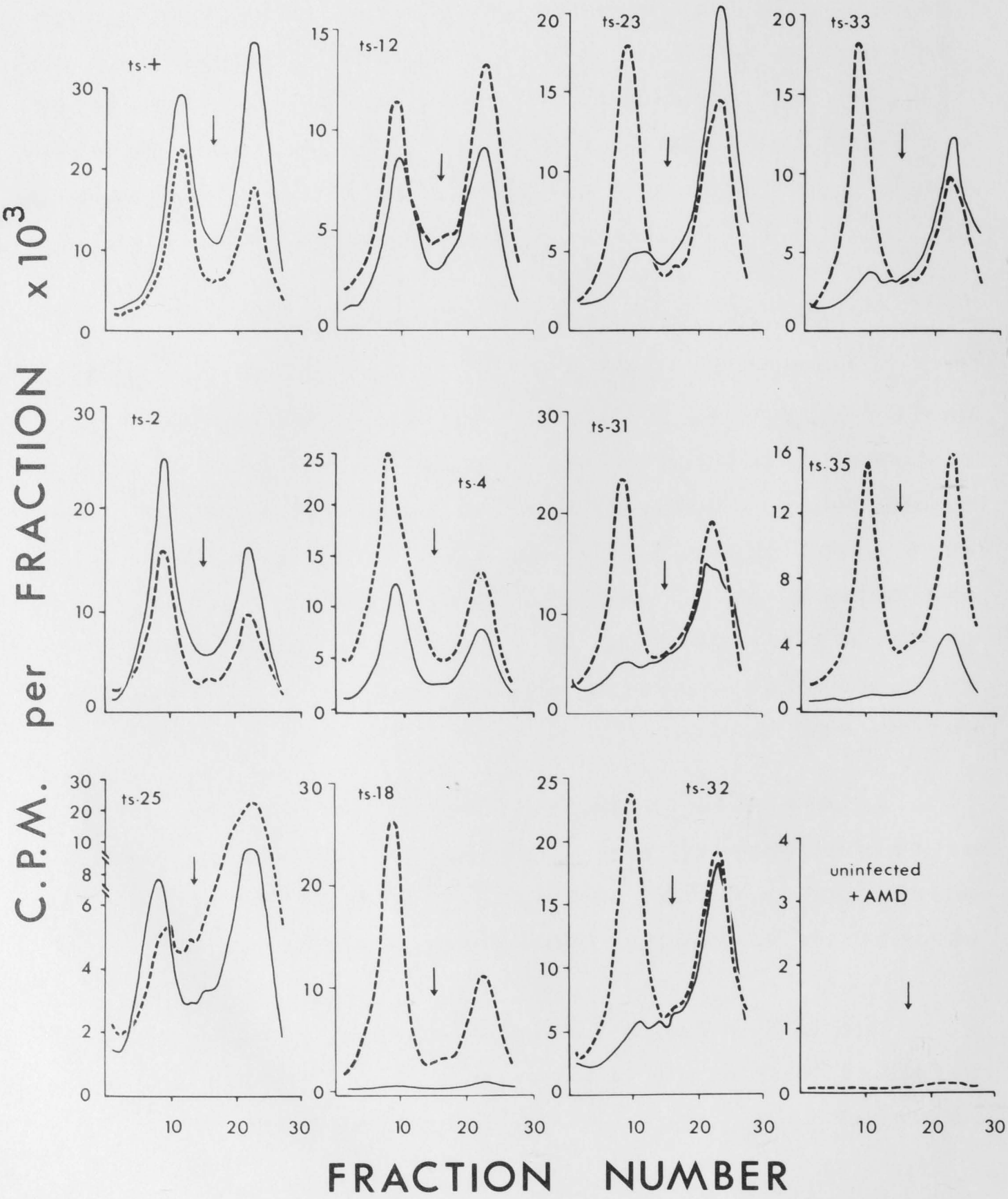


Figure 5-3. Nucleocapsid production by wild-type virus and RNA^+ mutants at 30^o and 38.5^o.

Infected cells were labeled with ^3H uridine and nucleocapsids were extracted and analysed as in figure 5-1. (--- = radio-activity at 30^o, ——— = radioactivity at 38.5^o).

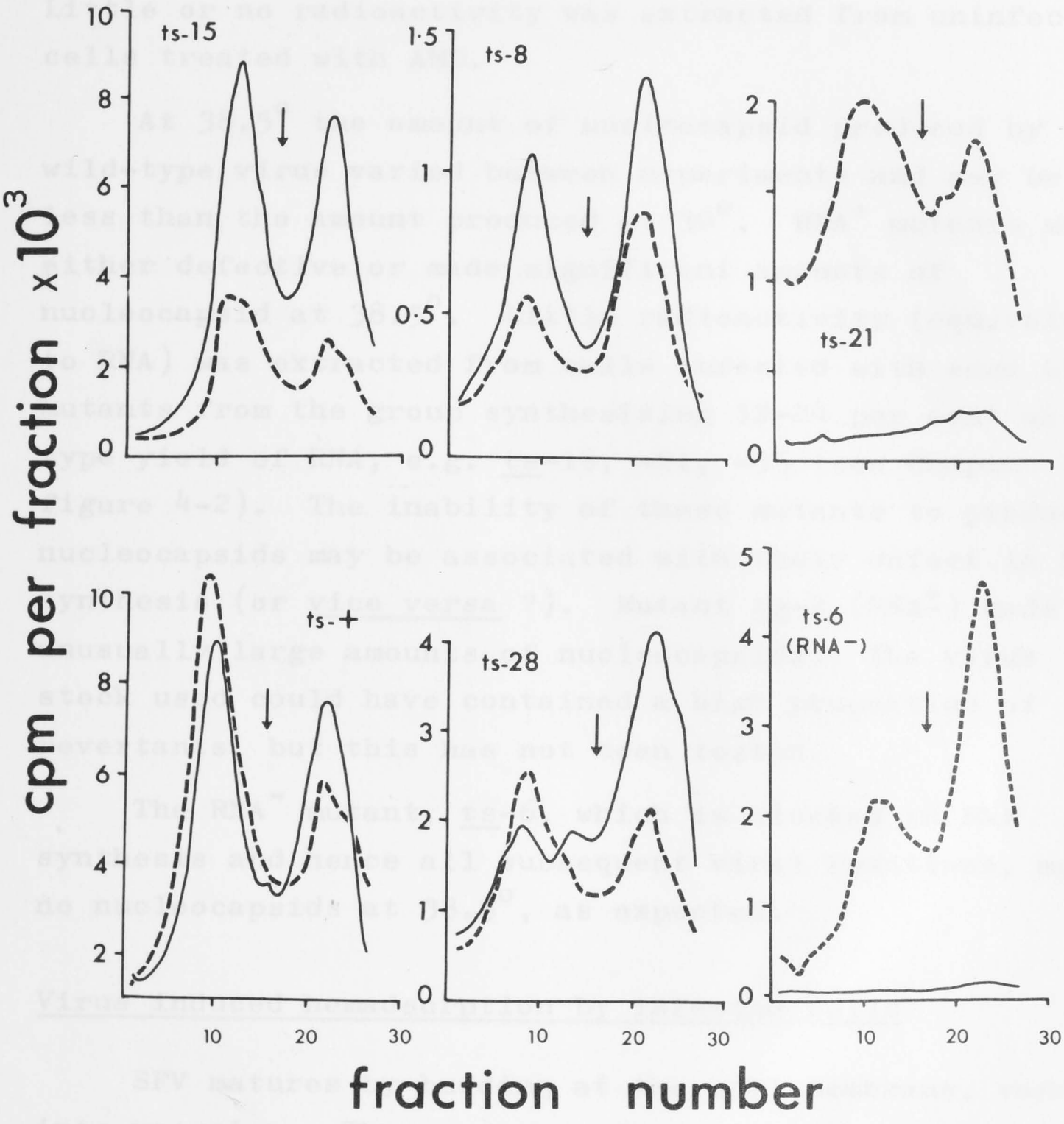


Figure 5-4. Nucleocapsid production by wild-type virus and ts mutants at 30° and 38.5°. Details as in figure 5-3. Mutants, except ts 6, are RNA^+ mutants.

At 30° all mutants produced nucleocapsids but, with most mutants, in lesser amounts than wild-type virus. Little or no radioactivity was extracted from uninfected cells treated with AMD.

At 38.5° the amount of nucleocapsid produced by wild-type virus varied between experiments and may be less than the amount produced at 30°. RNA^+ mutants were either defective or made significant amounts of nucleocapsid at 38.5°. Little radioactivity (equivalent to RNA) was extracted from cells infected with some RNA^+ mutants from the group synthesising 12-24 per cent wild-type yield of RNA, e.g. ts-18, -21, -35 (see Chapter 4, figure 4-2). The inability of these mutants to produce nucleocapsids may be associated with their defect in RNA synthesis (or vice versa ?). Mutant ts-2 ($RNA^{\pm}$) made unusually large amounts of nucleocapsids. The virus stock used could have contained a high proportion of revertants, but this has not been tested.

The RNA^- mutant, ts-6, which is blocked in RNA synthesis and hence all subsequent viral functions, made no nucleocapsids at 38.5°, as expected.

Virus induced hemadsorption by infected cells

SFV matures by budding at the cell membrane, rarely into vacuoles. The presence of viral membrane protein on the cell surface can be detected by the ability of infected cells to adsorb gander red blood cells, a property assumed to be of the same antigenic basis as hemagglutination of gander red blood cells by virions. Burge and Pfefferkorn (1967) developed a sensitive hemadsorption technique employing $^{51}CrO_4^{2-}$ labelled

TABLE 5-1. HEMADSORPTION INDUCED BY TS MUTANTS

TS MUTANT	CPM PER MONOLAYER INCUBATED AT: 30°	38.5°	HEMADSORBING CAPACITY AT 38.5°
6 (RNA^-)	5784	396	-
4 (RNA^+)	2556	132	-
23 (RNA^+)	1917	153	-
26 (RNA^+)	1225	100	-
15 (RNA^+)	2011	522	+
21 (RNA^+)	3632	1197	+
35 (RNA^+)	1615	436	+
WILD-TYPE	2768	2884	+
WILD-TYPE (+ ANTISERUM)	193	—	
UNINFECTED CELLS	550	370	

Cells were inoculated with ^{51}Cr-labeled gander red blood cells and kept at 4° for 1 hour. Unadsorbed red blood cells were washed off and those firmly attached were measured for radioactivity.

gander red blood cells for the detection of viral membrane protein on the surface of infected cells. The same technique was used to investigate hemadsorption induced by SFV ts mutants.

CE monolayers infected at 38.5° or 30° were assayed for hemadsorbing capacities after 7 hours or 8.5 hours of incubation (virus growth is slower at 30°, hence the longer incubation period). AMD was omitted to prevent premature death of cells. The experiment was done twice with similar results and the results of one experiment are presented in Table 5-1. Hemadsorption was virus-specific as the hemadsorbing capacity of cells infected with wild-type virus was completely neutralised by specific anti-SFV serum (exhaustively adsorbed with uninfected CE cells).

Cells infected with the RNA^- mutant ts-6 and 3 RNA^+ mutants (ts-4,-23,-26) induced significantly less hemadsorption at 38.5° than at 30°. These mutants presumably fail to produce sufficient membrane protein to induce hemadsorption. Three other RNA^+ mutants (ts-15,-21,-35) hemadsorbed more cells than the above mutants. Uninfected CE cells showed unusually high hemadsorbing levels, higher even than cells infected with some mutants at 38.5° or wild-type virus infected cells treated with antiserum. The hemadsorbing capacities at 38.5° of mutant infected cells were determined by comparing the radioactivity with that at 30°. If the radioactivity at 30° was more than 10 times that at 38.5°, the cells were scored as defective or '-'; if the difference was less than 10 fold, cells were scored as not defective or '+' (Table 5-1).

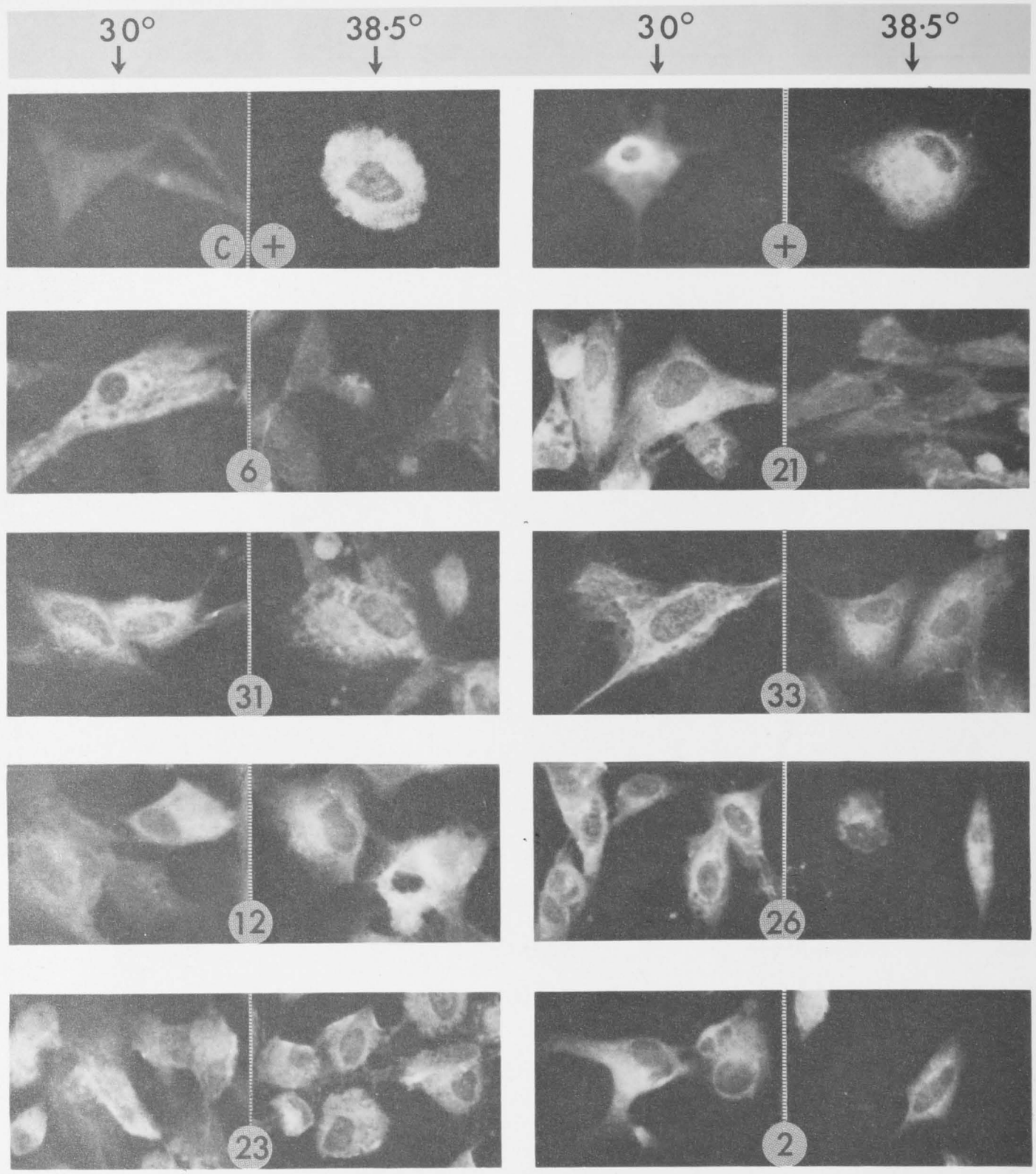


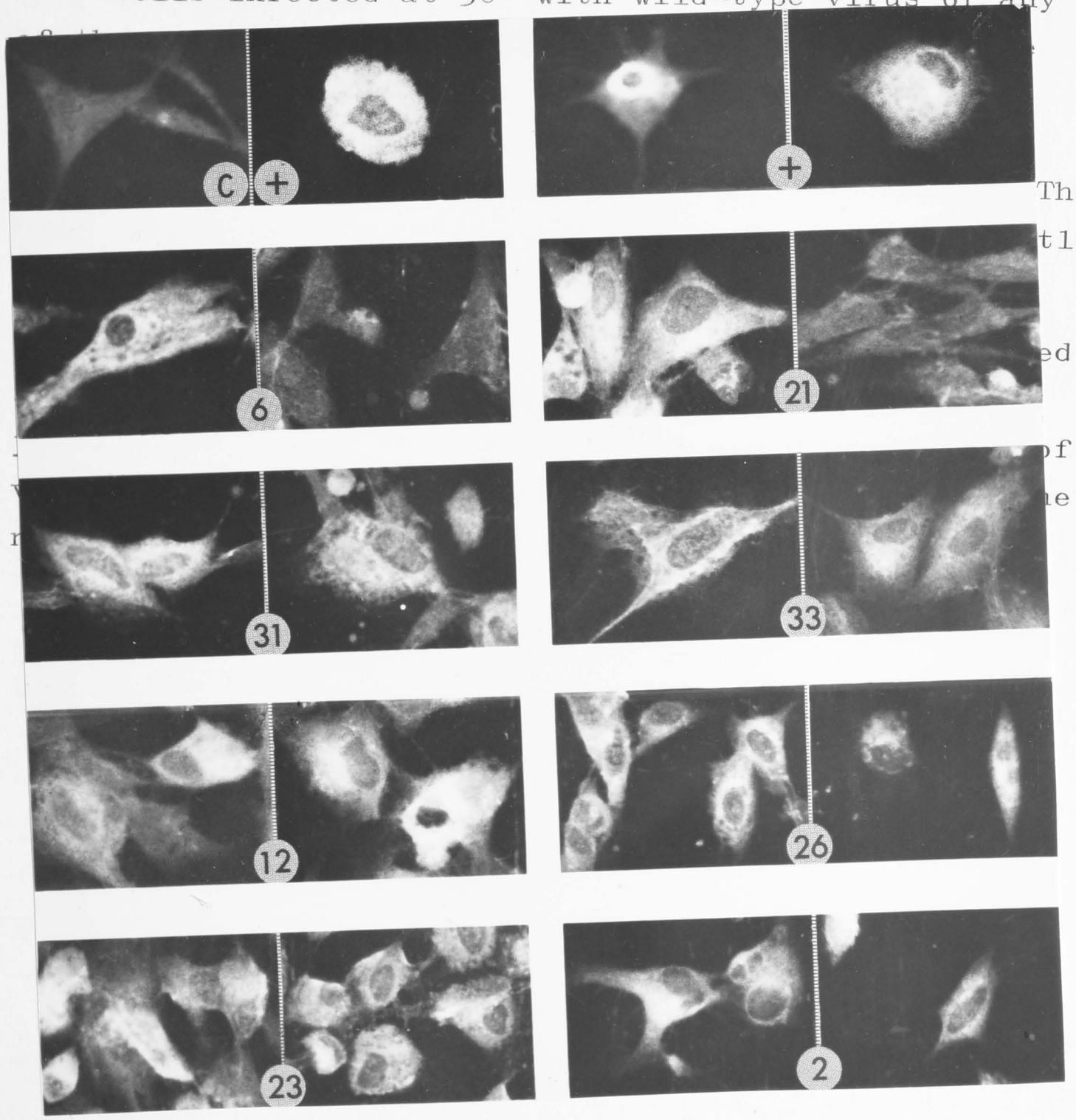
Plate 5-2. Detection of viral antigen with fluorescent antibody.

Cells infected with ts mutants or wild-type virus ('+') were tested for antigen produced after 8 hours incubation at 30° or 38·5°. Only one RNA⁻ mutant, ts-6, is shown. Uninfected cells are denoted by 'C'.

Detection of viral antigen with fluorescent antibody

Although incapable of producing infectious virus at 38.5°, mutants may be making antigens which could be detected by fluorescent antibody staining of infected cells.

Cells infected at 30° with wild-type virus or any



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Detection of viral antigen with fluorescent antibody

Although incapable of producing infectious virus at 38.5°, mutants may be making antigens which could be detected by fluorescent antibody staining of infected cells.

Cells infected at 30° with wild-type virus or any of the mutants showed strong cytoplasmic fluorescence after staining with fluorescent antibody (Plate 5-2). At 38.5°, the $RNA^{\pm}$ mutant and all RNA^{+} mutants except one (ts-21) induced fluorescence in infected cells. The RNA^{-} mutant ts-6, as expected, and ts-21, induced little or no fluorescence at 38.5°.

Often strong perinuclear fluorescence was observed in infected cells which otherwise looked normal (e.g. ts-+, Plate 5-2). This may reflect early production of viral antigens which are initially localised around the nucleus and later spreading over the cytoplasm.

TABLE 5-2. FUNCTIONAL DEFECTS OF SFV ts MUTANTS AT 38.5°

ts mutants	RNA phenotype	Production of Nucleocapsid	Viral * membrane	Antigen
Wild-type	+	+	+	+
6	-	-	-	-
2	±	+	ND	+
23	+	-	-	+
18,31,32 33,35	+	-	+	+
4,26	+	+	-	+
12,15 25,28	+	+	+	+
21	+	-	+	-

* Inferred from hemadsorption tests.

ND = not determined; + = not defective; - = defective.

DISCUSSION

The nucleocapsids of SFV, Sindbis virus and WEE were reported to be unable to form plaques but to contain infectious RNA (Friedman and Berezesky, 1967; Burge and Pfefferkorn, 1968; Yin and Lockart, 1968; and Sreevalsan and Allen, 1968). Attempts to demonstrate 42S RNA in my SFV nucleocapsids failed probably because of the extraction technique. Ribonuclease added at a concentration of 0.1 µg/ml to the nucleocapsid preparation before centrifugation did not alter the sedimentation coefficient of the nucleocapsid but probably effectively degraded the RNA in them. Similar observations were made by Sreevalsan and Allen (1968) who reported that very low concentrations of ribonuclease (e.g. 0.001 µg/ml) effectively gained access to and degraded the RNA in WEE nucleocapsids.

Subsequent to viral RNA synthesis, there are at least 2 important morphogenetic events in the replication of SFV. These are (i) the production of viral nucleocapsids, and (ii) the production of viral membrane protein in the cellular membrane to prepare it for the formation of virus particles by budding (Acheson and Tamm, 1967; and Friedman and Berezesky, 1967). The presence of nucleocapsids can be detected by analysing the cytoplasmic extract of infected cells in sucrose gradients and the presence of viral membrane protein in the cellular membrane can be detected by a hemadsorption test.

Summarising the results of these tests (Table 5-2), SFV RNA^+ mutants can be placed into 4 physiological groups:

(1) mutants defective in the production of nucleocapsids;

(2) mutants defective in the production of viral membrane protein;

(3) mutants which made both structural proteins but not infectious virus; and

(4) a mutant defective in the production of both structural proteins and may be a double-mutant.

At 30° and notably at 38.5°, most mutants induced lower levels of hemadsorption than wild-type virus. However, ts-6 (RNA^-) infected cells incubated at 30°, hemadsorbed more gander cells than the cells infected with wild-type virus. The significance of this is not known. The membrane protein of ts-6 may possess enhanced hemadsorbing activity.

Of the 4 mutants (ts-12,-15,-25,-28) which made both structural proteins at 38.5°, ts-12,-15 and -28 have virions which are heat labile at 50.5° (see Chapter 7). The proteins of these mutants may be altered in structure when compared with those of the wild-type virions. At the restrictive temperature these altered structural proteins may not be able to maintain a functional configuration to complete the replication cycle. The virions of ts-25 are heat stable (see Chapter 7); presumably the structural proteins are normal in structure. Mutant ts-25 may be the equivalent of ts-20 of Sindbis virus which is thought to be defective in a virus coded function needed for maturation of the virion (Burge and Pfefferkorn, 1968).

All the RNA^+ mutants, except one (ts-21), made in infected cells antigens which were detectable by fluorescent antibody staining. Antiserum to SFV was obtained from rabbits inoculated with live virus and in theory antibodies against both viral structural proteins and viral RNA polymerase (Polatnick et al, 1967) may be present. However, it is unlikely that the SFV antiserum contained significant amounts of antibodies against viral RNA polymerase because ts-21 induced no fluorescence while all the other mutants, which synthesise low levels of RNA like ts-21, induced fluorescence.

Assuming that the antiserum reacted mainly against viral structural proteins, then mutants which are defective in the production of either nucleocapsid or viral membrane protein, or of both, must be synthesising the polypeptides of these proteins if they induced fluorescence. The defect of these mutants may be in the assembly of the polypeptides to form nucleocapsids or viral membranes. The analysis of cytoplasmic extracts of infected cells by polyacrylamide gel electrophoresis may give an indication of the defects of these mutants; but this has not been done.

Cells infected with ts-21 showed no fluorescence even though viral membrane protein was made. It is possible that (i) fluorescence is caused mainly by the nucleocapsid protein, or (ii) the membrane protein of ts-21 is altered and does not react with antibodies.

CHAPTER 6

TIME OF EXPRESSION OF *ts* DEFECTS

INTRODUCTION

Pfefferkorn and Burge (1967) distinguished Sindbis ts mutants as 'early' or 'late' mutants. All the early mutants were RNA^- mutants which were defective in a function expressed early in the growth cycle (within 4 hours after infection) and this was presumed to be the synthesis of viral RNA polymerase. In contrast, RNA^+ mutants were found to be late mutants in which the ts defects were expressed late in the growth cycle (after 4 hours after infection). These defects were later identified as the synthesis of viral structural proteins and the maturation of virus particles (Burge and Pfefferkorn, 1968).

SFV ts mutants have been demonstrated to possess physiological defects (described in Chapters 4 and 5) similar to those of Sindbis virus ts mutants and were therefore expected to be defective in functions expressed at different periods of the growth cycle. The times at which the ts defects of SFV mutants were expressed were investigated by 2 types of experiments in which cells were transferred at early stages of the growth cycle (at intervals of 1 to 4 hours after infection) from the permissive temperature (28^o) to the restrictive temperature (38.3^o) ('shift-up' experiment); or from 38.3^o to 28^o ('shift-down' experiment). All the cultures were washed 4 hours after infection (the time at which progeny virus begins to be released from the cells) and the yields of virus assayed 8 hours after infection.

The types of result that can be expected from shift-up and shift-down experiments are as follows:

(a) Shift-up experiment

If the ts defect was expressed early (during first 4 hours of infection), then significant yields of virus should be produced at 38.3° after the shift-up, since the critical period has been bypassed by prior incubation at 28° for 4 hours. Conversely, if the ts defect was expressed late in infection (after 4 hours), then the mutants will not produce virus under shift-up conditions as the incubation temperature then would be 38.3°.

(b) Shift-down experiment

Mutants with an early defect will be inhibited in growth since the first 4 hours of incubation were at 38.3°. However, under the same conditions mutants with late defects should produce normal yields of virus since the critical period of the growth cycle (after 4 hours after infection) was at 28°.

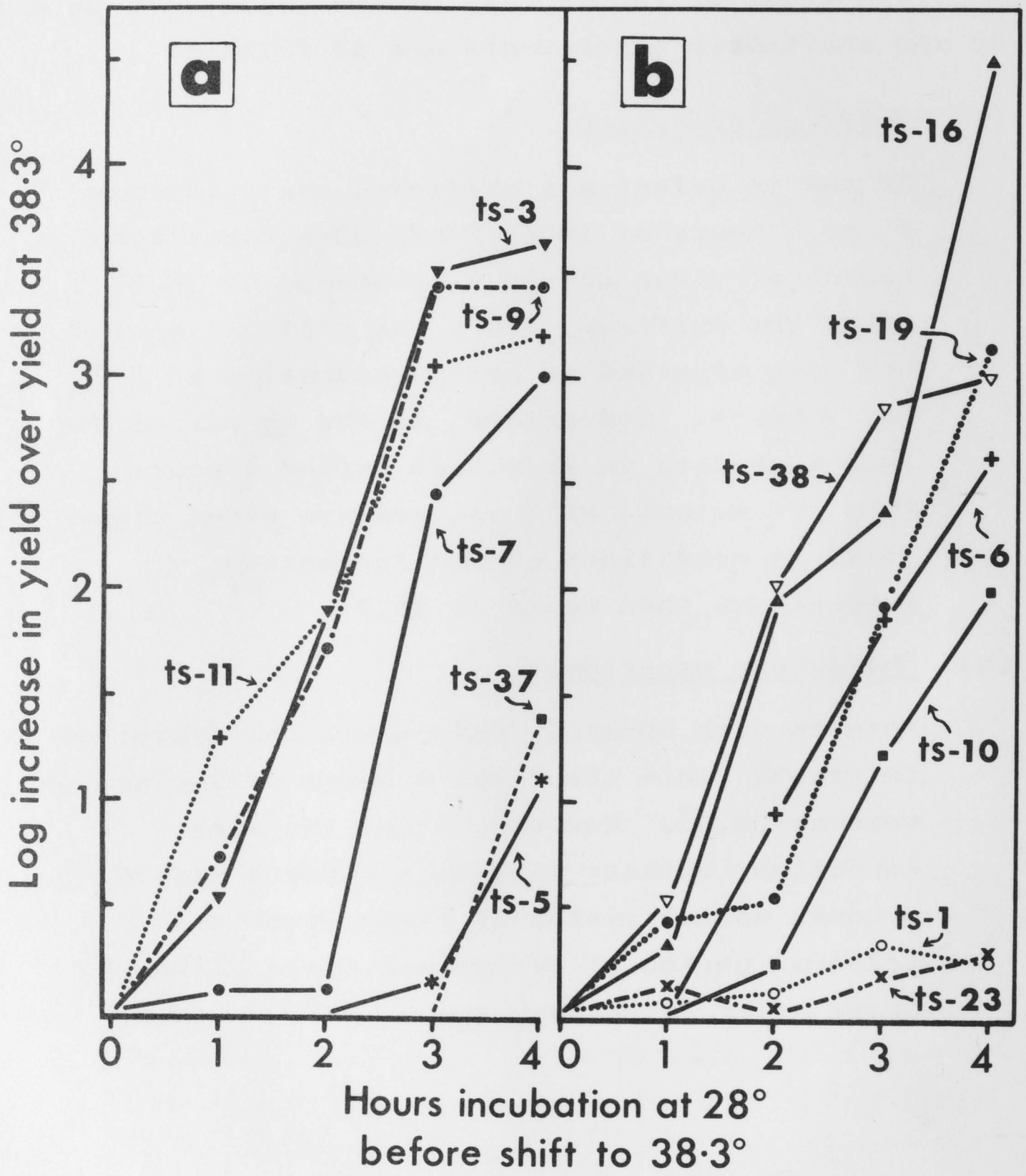


Figure 6-1. Temperature shift-up experiment. Minilayers were inoculated with virus and after a 2 hr adsorption period at 4°, the cells were washed and incubated at 28° in the presence of AMD. After 1-4 hr at 28°, samples were transferred to 38.3°. 4 hr after infection the medium of all samples was replaced with fresh medium containing AMD and virus produced during the next 4 hr was measured (duration of experiment was 8 hr). The increase in yield over the yield produced in the same period by infected cells incubated throughout at 38.3° was plotted. Only one RNA^+ mutant, ts-23, is shown, all the other mutants are RNA^- mutants.

	TS MUTANT	SHIFT-UP 28°C TO 38.3°C [a] YIELD SHIFT-UP / YIELD 28°	SHIFT-UP 28°C TO 38.3°C [a] YIELD SHIFT-UP / YIELD 38.3°	SHIFT-DOWN 38.3°C TO 28°C [b] YIELD SHIFT-DOWN / YIELD 28°	SHIFT-DOWN 38.3°C TO 28°C [b] YIELD SHIFT-DOWN / YIELD 38.3°
RNA⁻	1	[c] 0.32 x 10^{-2}	1.77	[d] 0.15 x 10^{-2}	1.70
	5	0.33 x 10^{-2}	26.00	0.94 x 10^{-3}	4.48
	37	0.38 x 10^{-1}	25.00	0.16 x 10^{-2}	0.15
	7	0.06	1.13 x 10^{3}	0.56 x 10^{-3}	20.00
	9	0.48	2.00 x 10^{3}	0.51 x 10^{-3}	1.20
	10	0.17	56.00	0.14 x 10^{-2}	0.38
	11	0.37	1.51 x 10^{3}	0.13 x 10^{-2}	26.47
	3	0.88	4.22 x 10^{3}	0.12 x 10^{-2}	3.50
	6	1.07	4.09 x 10^{2}	0.75 x 10^{-3}	0.46
	16	0.80	3.24 x 10^{4}	0.96 x 10^{-4}	1.43
	19	0.62	1.41 x 10^{3}	0.75 x 10^{-3}	0.07
	38	1.25	9.90 x 10^{2}	0.46 x 10^{-2}	3.10
RNA±	2	0.31 x 10^{-3}	4.23	0.32	1.30 x 10^{3}
RNA⁺	4	0.53 x 10^{-2}	1.06	0.25	35.29
	14	0.20 x 10^{-1}	0.56	0.33	7.00
	18	0.15 x 10^{-3}	4.07	0.15	4.75 x 10^{2}
	21	0.16	3.54	0.38	ND
	23	0.25 x 10^{-2}	2.00	0.26	2.14
	25	0.30 x 10^{-1}	1.60	0.40	1.04 x 10^{2}
	26	0.47 x 10^{-1}	1.65	0.46	80.00
	30	0.12 x 10^{-2}	0.92	0.46	2.05 x 10^{2}
	12	0.14 x 10^{-1}	0.51	1.05	22.45
	13	0.14 x 10^{-1}	0.61	0.56	ND
	15	0.25 x 10^{-2}	0.55	0.63	64.29
	27	0.46 x 10^{-2}	0.45	1.07	ND
	28	0.17 x 10^{-2}	5.00	1.00	1.66 x 10^{3}
	31	0.27 x 10^{-2}	1.43	0.53	9.00 x 10^{2}
	32	0.41 x 10^{-2}	1.85	0.91	5.47 x 10^{2}
	33	0.92 x 10^{-2}	1.62	0.62	1.14 x 10^{2}
	35	0.33 x 10^{-2}	0.12	0.51	5.69 x 10^{2}
	36	0.31 x 10^{-1}	5.63	0.83	1.11 x 10^{2}
	+	0.77	0.71	0.97	0.70

Table 6-1. Temperature shift-up and shift-down experiments.

a. Cells infected with wild-type virus or mutants were incubated for 4 hr at 28° and then transferred to 38.3° . The virus produced during the next 4 hr at 38.3° was compared with the yield produced in the same period by infected cells incubated throughout at 28° or 38.3°.

b. Infected cells were incubated for 4 hr at 38.3° before the transfer to 28° and the virus produced during the next 4 hr at 28° was compared with the yield produced in the same period by infected cells incubated throughout at 28° or 38.3° .

c. Yield shift-up or, d. yield shift-down expressed, in a summarised form, as % of the yield at 28°.

RESULTS

The kinetics of expression of the ts defects, shown in figures 6-1 and 6-2, were plotted by determining the 8-hour yields obtained with 1 to 4 hours incubation at 28° (in shift-up) or at 38.3° (in shift-down) before the temperature shift.

The yields from shift-up and shift-down experiments were then compared quantitatively with the yields from cells held throughout the experiments at either the permissive or the restrictive temperatures. In this case the temperature shift was performed 4 hours after infection. At this time all the cells were washed and the viral yields were assayed 8 hours after infection.

Temperature shift-up experiments

Most RNA^- mutants (except ts-1,-5,-37) produced large yields of virus in shift-up experiments. Mutants ts-5 and ts-37 produced some virus at 38.3° but only after 4 hours prior incubation at 28° (figure 6-1a), and this yield represented only about 4 per cent of the normal yield at 28°. With the other RNA^- mutants (except ts-1), e.g. ts-3 and ts-6, as the incubation at 28° was increased to 1 hour or more, subsequent viral production at 38.3° increased in an almost exponential fashion (figure 6-1). When the incubation at 28° was increased to 4 hours, subsequent viral production at 38.3° was normal or nearly so (Table 6-1).

One RNA^- mutant, ts-1, the $RNA^{\pm}$ mutant ts-2 and all RNA^+ mutants (e.g. ts-23 in figure 6-1b) produced very

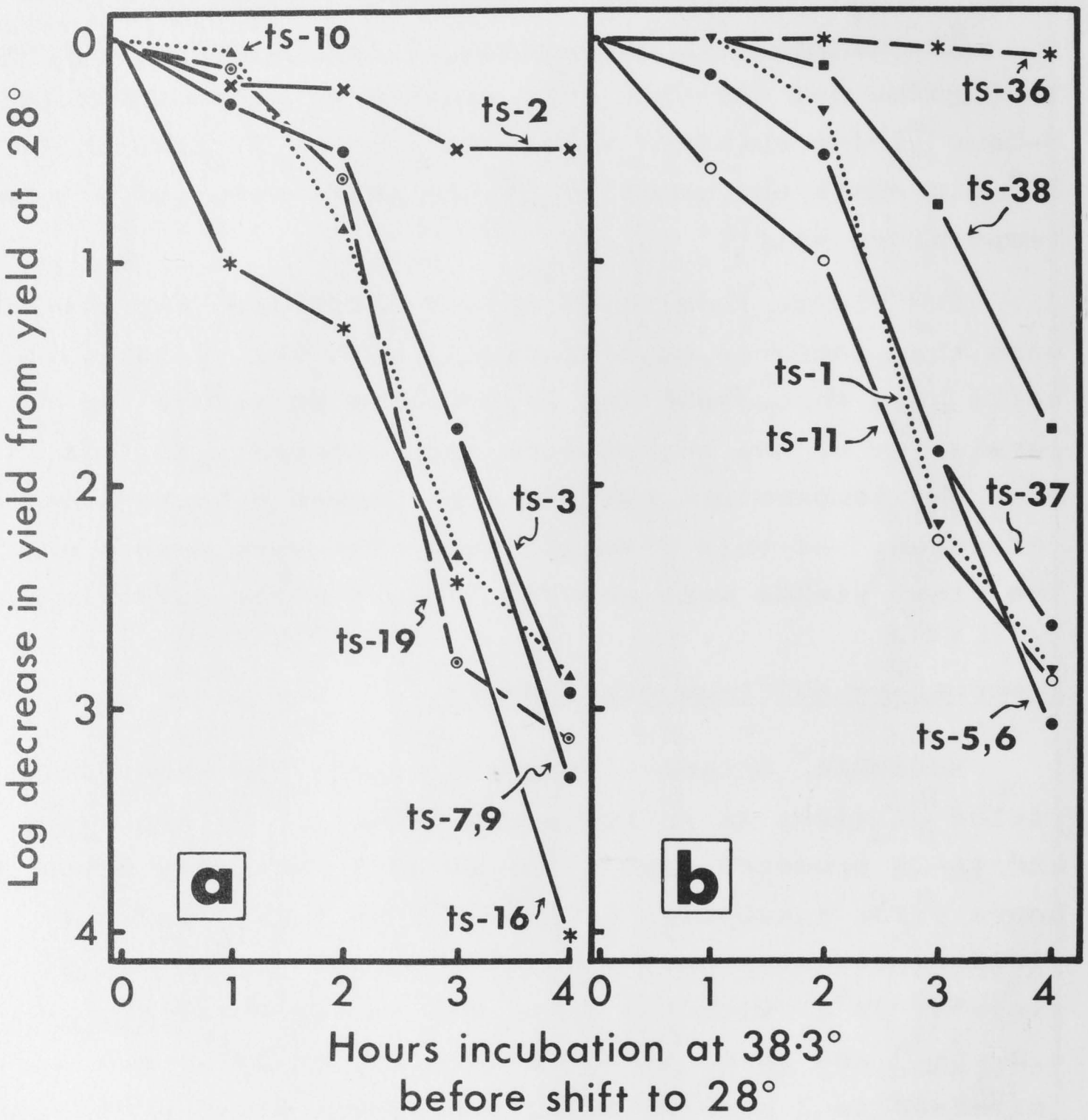


Figure 6-2. Temperature shift-down experiment. Cells infected as in figure 6-1 were initially incubated at 38.3°. After 1-4 hr at 38.3°, samples were transferred to 28° and the virus produced from 4-8 hr after infection was measured, as in figure 6-1. The decrease in yield from the yield produced during the same period by infected cells incubated throughout at 28° was plotted. ts-2 is a $RNA^{\pm}$ mutant, ts-36 is RNA^{+} and all the others are RNA^{-} mutants.

little virus (i.e. less than 3 per cent of normal yield) in shift-up experiments (Table 6-1).

Thus the RNA^- mutants (except ts-1,-5,-37) appear to have a critical period early in the growth cycle.

Temperature shift-down experiment

In shift-down experiments the $RNA^{\pm}$ and RNA^+ mutants produced normal or nearly normal yields (Table 6-1). The critical periods of these mutants therefore seemed to be late in the growth cycle.

With increasing periods of incubation at 38.3^o, decreasing yields of RNA^- mutants were obtained with subsequent incubation at 28^o (figure 6-2). This probably reflects inhibition of viral replication at 38.3^o. For example, cells infected for 1 hour at 38.3^o and then transferred to 28^o would have 7 hours of incubation at 28^o since the total duration of the experiment was 8 hours. Similarly, cells infected for 2, 3 and 4 hours at 38.3^o before the transfer to 28^o would subsequently receive 6, 5 and 4 hours, respectively, of incubation at 28^o. Therefore, the final yields measured in shift-down experiments were the yields of 7, 6, 5 and 4 hours growth at 28^o and would explain the decreasing yields of RNA^- mutants shown in figure 6-2.

This apparent inhibition of viral replication at 38.3^o was further investigated with 2 RNA^- mutants, ts-6 and ts-16. Three sets of cells were infected with each mutant. One set of cells was incubated for 8 hours at either 38.3^o or 28^o. The other set was incubated for 4 hours at 38.3^o, then transferred to 28^o and incubated

TABLE 6-2. REVERSIBILITY OF INHIBITION OF YIELDS OF RNA⁻ MUTANTS INCUBATED AT 38·3°

TS	YIELD PFU PER ML		
	[a] 8 HR AT 38·3°	[b] 8 HR AT 28°	[b] 4 HR AT 38·3° THEN 8 HR AT 28°
6	14×10^4	33×10^6	23×10^6
16	43×10^2	64×10^6	5×10^6

[a] Yield from fourth to eighth hr incubation at 38·3°.

[b] Yield from fourth to eighth hr incubation at 28°.

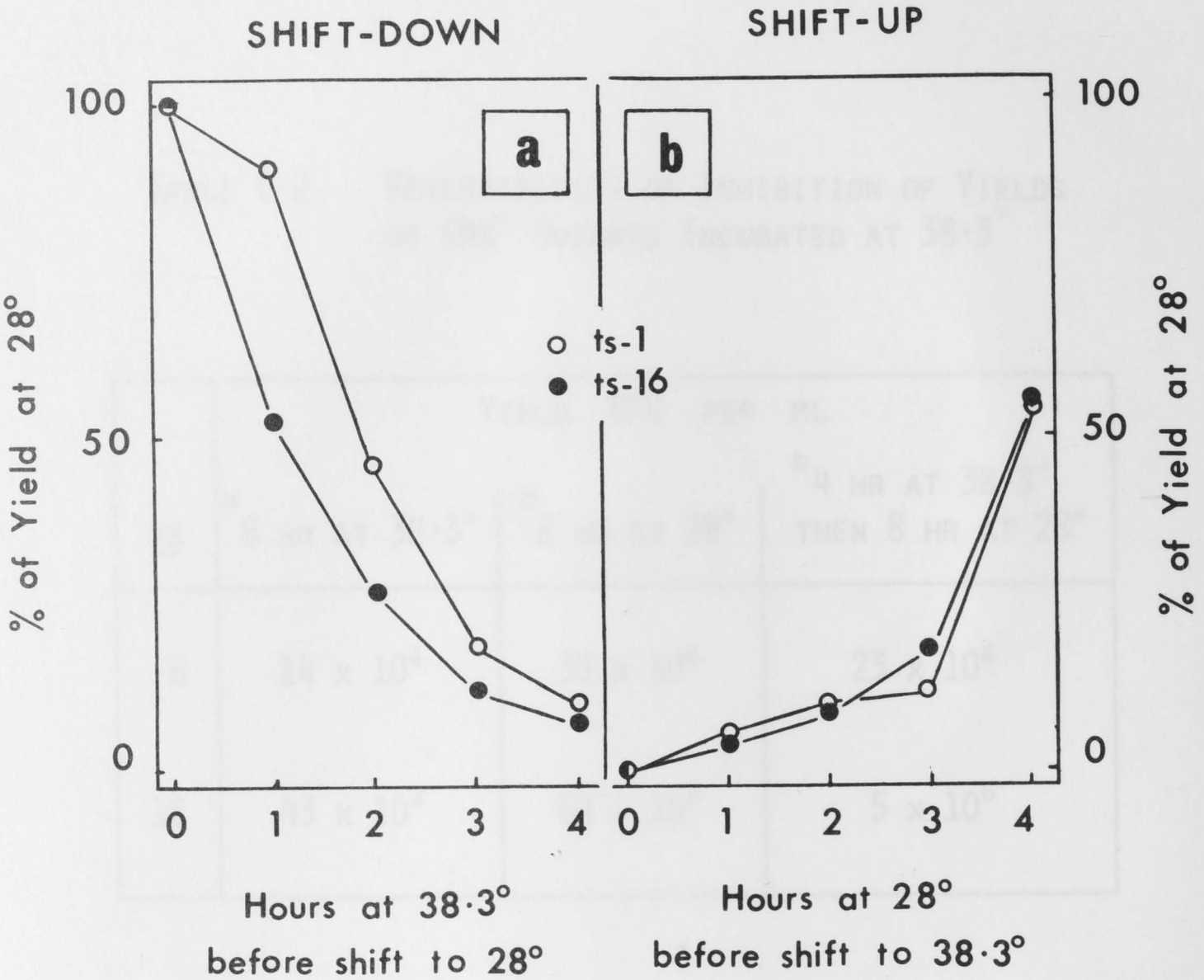


Figure 6-3. Synthesis of RNA by 2 RNA^- mutants in temperature shift experiments.

Experimental conditions are as described in figure 6-1 and figure 6-2 except that instead of measuring infectivity, ^3H uridine (added to the growth medium) incorporated from 4.5-8 hr after infection was measured and expressed as a percentage of the value obtained with infected cells incubated throughout at 28°.

for another 8 hours. The results of this experiment are presented in Table 6-2. The viral yields from cells incubated at 28° with or without prior incubation at 38.3° were significantly different from the yield from cells incubated throughout at 38.3°. It is concluded that at 38.3° the replication of RNA⁻ mutants was inhibited and that this inhibition was almost completely reversible when conditions were returned to normal.

RNA synthesis by RNA⁻ mutants

Three RNA⁻ mutants, ts-1,-5,-37, were also blocked in a late function. One of them, ts-1, was studied further by investigating RNA synthesis under temperature shift conditions. These experiments were performed as before except that ^{3}H uridine incorporation from 4.5 to 8 hours after infection was measured instead of infectivity. The RNA⁻ mutant, ts-16, which made a normal yield under shift-up conditions, was included for comparison.

In shift-up experiments, both ts-1 and ts-16 had identical patterns of incorporation of radioactivity (equals RNA synthesis) (figure 6-3b). Significant amounts of RNA were synthesised by the two mutants late in the growth cycle (4.5 to 8 hours) at 38.3° under shift-up conditions. This implies that viral RNA polymerase, once made at 28°, was stable and functional at 38.3°.

The RNA synthesised by ts-1 in the shift-up experiment was infectious (Table 6-3). Significantly more infectious RNA was extracted from 'shift-up cells'

TABLE 6-3. SYNTHESIS OF INFECTIOUS RNA BY ts-1 IN SHIFT-UP EXPERIMENT

INCUBATION	PFU PER CULTURE
7 HR AT 28°	52×10^4
4 HR AT 28°, THEN 3 HR AT 38.3°	68×10^4
7 HR AT 38.3°	30×10^3

RNA was extracted from infected cells with SDS/phenol (see Materials and Methods) and assayed for infectivity at 28°.

than from cells infected throughout at 38.3° or at 28°; in the latter case it probably resulted from more rapid RNA synthesis at 38.3° (i.e. once initiated at 28°) than at 28°. It seems that ts-1 is defective in another function located after the synthesis of infectious RNA, i.e. ts-1 is probably a double mutant.

The decreasing yields of RNA obtained in shift-down experiments (figure 6-3a) probably reflects inhibition of RNA synthesis operating on the same basis as that in figure 6-2.

TABLE 6-4. SUMMARY OF TEMPERATURE SHIFT EXPERIMENTS[1]

GROUP	TS MUTANT	RNA YIELD[2] AT 38.3° COMPARED WITH WILD-TYPE VIRUS	YIELD SHIFT-UP / YIELD AT 28°	YIELD SHIFT-DOWN / YIELD AT 28°	CRITICAL PERIOD OF GROWTH CYCLE
A	3, 6	2 - 3% (RNA-)	88 - 107%	0.1%	EARLY
B	1, 5, 37	2 - 4% (RNA-)	0.3 - 0.4%	0.1 - 0.2%	LATE (?)
C	27, 28	74 - 80% (RNA+)	0.2 - 0.5%	100 - 107%	LATE

1 SUMMARISED FROM TABLE 6-1. ONLY A FEW REPRESENTATIVE MUTANTS ARE SHOWN IN GROUPS A AND C.

2 FROM TABLE 4-1, SEE CHAPTER 4.

DISCUSSION

The response of $RNA^{\pm}$ and RNA^{+} mutants to temperature shift experimentation is straightforward. The only critical period of their growth cycle is late in infection and is subsequent to viral RNA replication (Table 6-4). This is in agreement with the results of physiological tests (reported in Chapter 5) in which the mutants were shown to be defective in the production of viral structural proteins or in maturation function(s). Martin (1969) reported that 2 SFV RNA^{+} mutants (ts-15 and ts-28) synthesised functional RNA polymerase at the restrictive temperature. He concluded that these mutants are defective in function(s) expressed subsequent to viral RNA polymerase synthesis and hence RNA synthesis.

Some RNA^{-} mutants, e.g. ts-3 and ts-6 (Table 6-4), produced normal yields of virus under shift-up conditions. This suggests: (i) that the ts defect of these mutants is located early in the growth cycle and a gene product is not made at 38.3°; (ii) that once synthesised at 28°, the gene product is stable and functional at 38.3°. Thus, these RNA^{-} mutants seem to be defective in the initiation of synthesis of the early gene product and hence viral RNA synthesis; and (iii) that either saturation amounts of the gene product are made early in the growth cycle to ensure normal production of virus later in the growth cycle, or that once initiated, the synthesis of the gene product can occur at 38.3°. This early gene product is presumed to be viral RNA polymerase (Pfefferkorn and Burge, 1967; Martin, 1969).

Martin (1969), studying the same SFV RNA$^-$ mutants (ts-3 and ts-6), was unable to detect any viral RNA polymerase activity in cells infected with these mutants at the restrictive temperature. The polymerases of these mutants were stable and functioned normally when tested both *in vitro* (testing at 42^o the activity of polymerase made at 28^o) and *in vivo* (testing polymerase activity before and after a shift-up experiment). Martin arrived at the conclusion that in cells infected with these mutants, viral RNA polymerase was not synthesised at the restrictive temperature, but that once synthesised at the permissive temperature, the polymerase was stable and functional. His results are in agreement with and supplement my physiological studies.

The nature of the defects of the 2 RNA$^-$ mutants, ts-5 and ts-37, are not known but a few explanations can be offered: (i) the RNA polymerase of these mutants may be temperature-sensitive, i.e. it is unable to assume or maintain a functional configuration at 38.3^o but functions normally at 28^o. Martin (1969) reported that the polymerase of ts-5 made at the permissive temperature was functional, but at a reduced efficiency, when tested at the restrictive temperature. He concluded that the ts defect of ts-5 cannot be equated with its slightly defective polymerase; (ii) it is likely that both ts-5 and ts-37 are double mutants as their virions are heat labile when compared with wild-type virus (see Chapter 7); and (iii) ts-5 and ts-37 may be single mutants containing defective structural proteins which interfere with viral RNA synthesis at the restrictive temperature. Such mutants have been reported for poliovirus ts mutants (Cooper *et al*, 1969).

Mutant ts-1 (Table 6-4), unlike ts-5 and ts-37, has heat stable virions (see Chapter 7). It is probably a double mutant which is defective in the initiation of synthesis of both RNA polymerase and structural proteins.

In conclusion, most RNA^- mutants are 'early' mutants and RNA^+ mutants are 'late' mutants in which the ts defects are expressed before or after 4 hours of infection, respectively. Sindbis virus ts mutants possess functional defects similar to those of SFV mutants and could also be distinguished as early or late mutants (Pfefferkorn and Burge, 1967 and 1968).

CHAPTER 7

DEFECTS IN THE VIRIONS OF *ts* MUTANTS

INTRODUCTION

At the molecular level, mutants appear to result from a base change (spontaneous or induced) in the genetic material. The new base causes an alteration in the codon for a particular amino-acid so that a novel amino-acid is substituted in its place. With temperature-sensitive mutants, it is postulated that the resulting protein may be unable to assume or maintain a functional configuration at the restrictive temperature. At the permissive temperature this protein may function normally. If the novel amino-acid is located in a structural protein, then the mutant virion may, but need not, differ from wild-type virion in some measurable biological properties other than the temperature-sensitivity of viral development. Two properties of the virions of SFV ts mutants were investigated: (i) heat stability of infectivity at various temperatures, and (ii) hemagglutinating activity.

Burge and Pfefferkorn (1966a) found a close correlation between the RNA phenotypes of Sindbis ts mutants and their heat stability (of infectivity) at 60^{o}. All the RNA^{+} mutants, except ts-20, were heat labile when compared with the parental strain. Loss of infectivity at 60^{o} probably resulted from denaturation of viral protein(s) and Burge and Pfefferkorn suggested that the RNA^{+} mutants (except ts-20) have defective structural protein(s). The mutant ts-20 was heat stable and Burge and Pfefferkorn (1968) postulated that it was

defective in the maturation of the virion which, once formed, was heat stable. RNA^- mutants were as heat stable as the parental strain and were therefore presumed to be defective in non-structural protein(s) required for viral RNA synthesis.

The defects in the structural proteins of Sindbis virus ts mutants may not be the cause of the inability of the mutants to grow at the restrictive temperature, for the strain of Sindbis virus from which the parental strain of the mutants was derived was more heat labile at 60^o than any of the ts mutants; but it grew normally at the restrictive temperature.

Changes in the hemagglutinating activity of Sindbis virus ts mutants were examined by Yin and Lockart (1968). They reported that one out of 3 mutants tested was defective in hemagglutination, a property of the spikes of the viral envelope (Osterrieth, 1966).

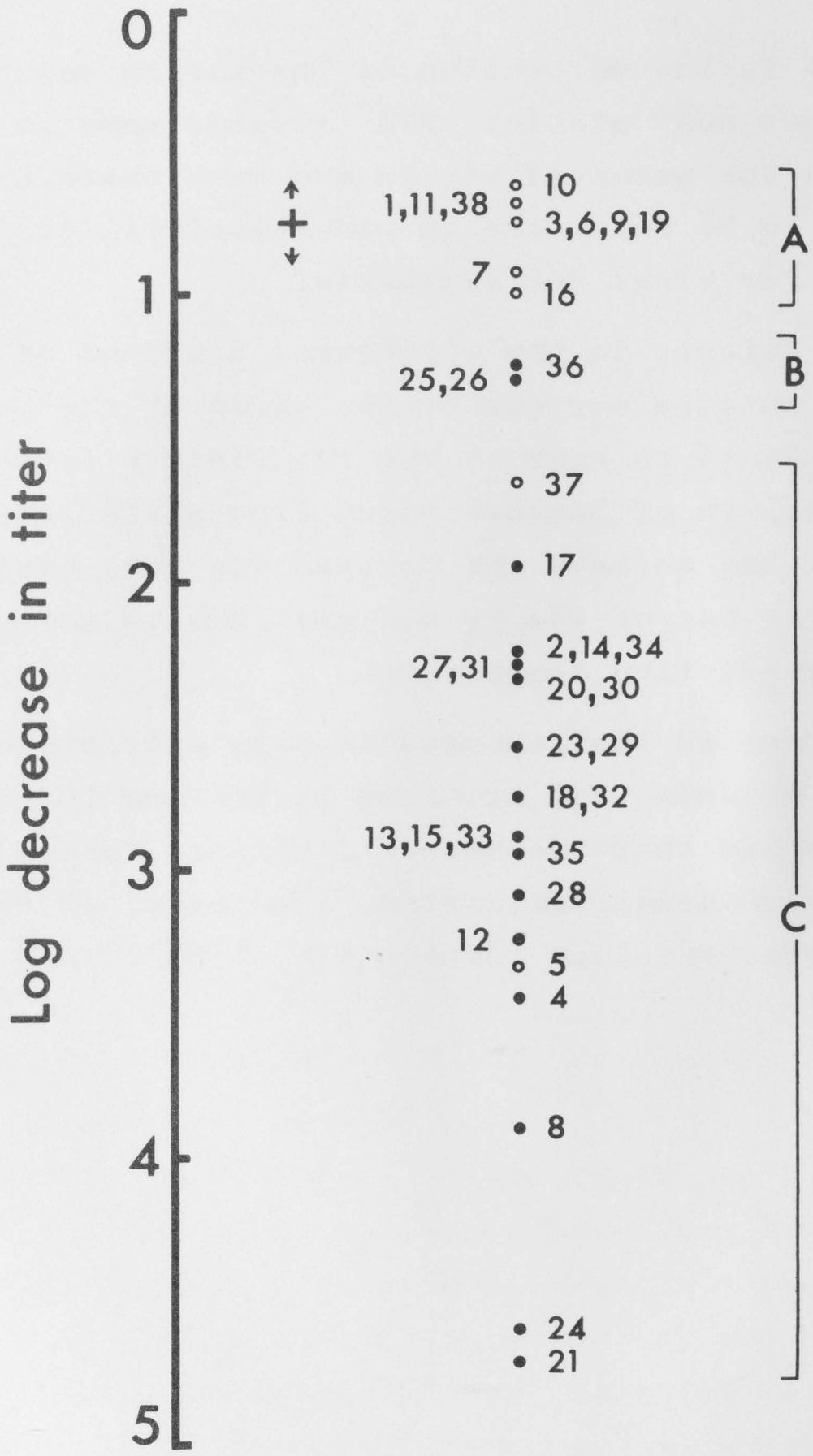


Figure 7-1. Heat inactivation of viral infectivity at 50·5° for 8 minutes.

The range of wild-type virus ('+') inactivation from 6 experiments is indicated by arrows. The mutants were placed into 3 arbitrary groups (A,B & C) according to their heat stability.

o = RNA^- mutants; • = $RNA^{\pm}$ and RNA^+ mutants.

Heat stability of viral infectivity

Methods

Virus in growth medium was diluted with an equal volume of gelatin saline and incubated at the restrictive temperature, or at the permissive temperature, or at 50.5°. The treated virus was then assayed for infectivity at the permissive temperature.

Inactivation at high temperatures, e.g. 50.5°, probably results from denaturation of protein. The nature of inactivation at low temperatures, e.g. 30°, is not clear and may involve viral RNA (Ginoza, 1968).

RESULTS

Heat stability of infectivity at 50.5°

Treatment of wild-type virus at 50.5° for 8 minutes resulted in a mild inactivation (less than one log drop) of infectivity and this temperature was chosen to test the mutants. All the mutants were tested in 4 main experiments which are collated in figure 7-1. Most RNA^- mutants appeared to be as heat stable as wild-type virus while most RNA^+ mutants were heat labile. An analysis of the standard error of the differences in heat stability of the 3 arbitrary groups in figure 7-1 showed that: (i) within group A, wild-type virus and the RNA^- mutants were not significantly different in heat stability at the 1 per cent level; (ii) The heat stability of groups A, B and C were significantly different from each other at the 1 per cent level. Group A mutants are considered

TABLE 7-1. HEAT STABILITY OF WILD-TYPE REVERTANTS OF HEAT LABILE ts MUTANTS *

VIRUS	LOG DECREASE IN TITER AFTER 8 MIN. AT 50.5°	E.O.P. AT 38.5°
WILD-TYPE	0.79	0.81
ts-18	3.02	$< 1 \times 10^{-5}$
REVERTANT I	0.40	0.78
REVERTANT II	0.66	0.51
ts-32	3.02	1.43×10^{-5}
REVERTANT I	0.78	0.47
REVERTANT II	0.78	0.67
ts-33	3.51	7.70×10^{-4}
REVERTANT I	2.64	0.41
REVERTANT II	1.14	0.54

* Wild-type revertants were selected for their ability to produce plaques at 38.5°. These revertants have e.o.p. at 38.5° (ratio of titer assayed at 38.5° to that at 30°) comparable to that of wild-type virus. Heat inactivation of viral infectivity was performed as in fig. 7-1.

as heat stable, group C mutants as heat labile and group B mutants are only slightly more heat labile than wild-type virus.

Heat stability of infectivity at 50.5° of wild-type revertants of heat labile mutants

If heat lability of infectivity is associated with the inability of heat labile mutants to grow at 38.5°, then revertants of these mutants which are able to grow at 38.5° are expected to be heat stable. Revertants of heat labile ts mutants were selected by plating the mutant stocks at 38.5°; clear wild-type size plaques were picked and stocks of these cloned revertants were prepared as described in Chapter 2.

Two revertants (which produced plaques normally at 38.5° and had e.o.p. at 38.5° comparable to that of wild-type virus) of each of 3 heat labile RNA^+ mutants were tested, in parallel with the original mutants and wild-type virus, for heat stability at 50.5°. As shown in Table 7-1, revertants of both ts-18 and ts-32 regained the full heat stability of wild-type virus. Revertants of ts-33, although significantly more heat stable than ts-33 itself, did not regain the full heat stability of wild-type virus. The implications of these observations are discussed below.

Heat stability of some mutants at the permissive and restrictive temperatures

A few mutants were examined for stability of infectivity at both 30° and 38.5°. It is clear that lability of infectivity at 38.5° could be associated

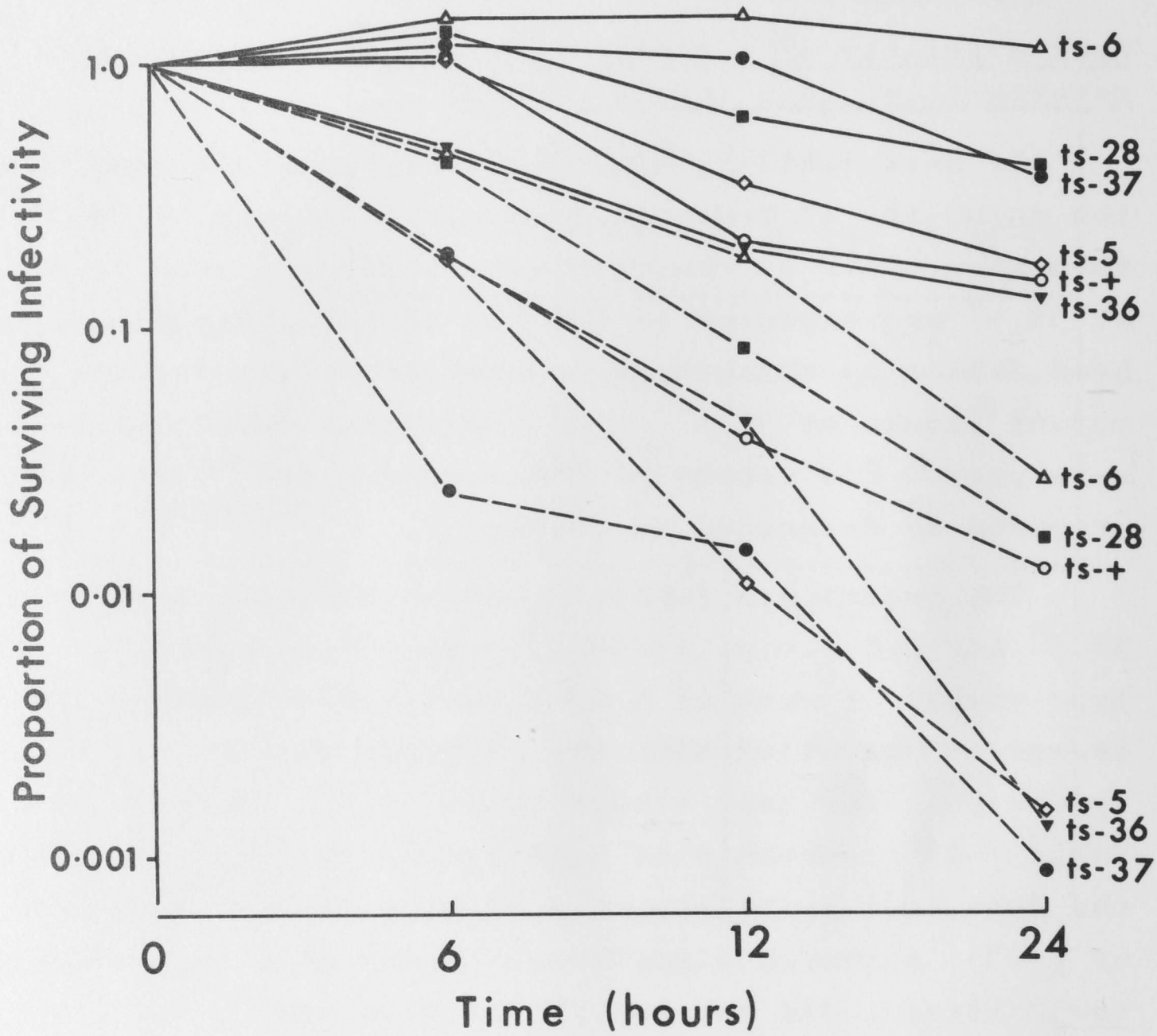


Figure 7-2. Heat stability of mutants at 30° (——) and at 38.5° (-----).

At intervals, 0.1 ml samples were removed and diluted immediately in ice-cold gelatin saline for assay of residual infectivity. ts-5, ts-6 and ts-37 are RNA^- mutants, the others are RNA^+ mutants.

with the temperature-sensitivity of growth of the mutants at this temperature. The same techniques were used as for testing heat stability at 50.5°.

At 30° there seemed to be no marked differences in the kinetics of inactivation of mutants or wild-type virus (figure 7-2). The slight increase in titer of most viruses at 6 hours probably resulted from disaggregation of clumps of virions.

At 38.5° differences in heat stability of the mutants were evident. Mutants ts-6 and ts-28 seemed to be as heat stable as wild-type virus while ts-5,-36 and -37 appeared heat labile. It is notable that ts-36 (which is relatively heat stable at 50.5°) is labile at 38.5°, and vice versa for ts-28. The implication of this is discussed below.

Changes in the hemagglutinating activity of mutants

If the mutation affected the configuration of the hemagglutinin, then changes in the hemagglutinating activity of the mutants would be expected. One RNA^- and 4 RNA^+ mutants were tested for changes in hemagglutination (HA).

Virus stocks prepared at 30° were centrifuged at 9,000g for 10 minutes to remove cellular debris. Virus was sedimented by centrifuging the supernatant at 145,000g for 70 minutes. The viral pellet was then extracted for hemagglutinins as described in Chapter 2.

Stocks of 2 mutants, ts-6 (RNA^-) and ts-23 (RNA^+) like wild-type virus, had high HA to PFU ratios (Table 7-2) and presumably contained normal hemagglutinins.

TABLE 7-2. HEMAGGLUTINATION OF VIRUS GROWN AT 28°

TS	HEMAGGLUTINATION UNITS PER 1×10^8 PFU*
6 (RNA^-)	92
4 (RNA^+)	6
21 (RNA^+)	5
26 (RNA^+)	12
23 (RNA^+)	42
+ (RNA^+)	36

* Virus was pelleted, its titer calculated by determining the titer remaining in the supernatant, extracted for antigen and tested for hemagglutination of gander red blood cells at pH 6.4.

The other 3 RNA^+ mutants produced significantly lower HA to PFU ratios than wild-type virus. It is possible that these 3 mutants produce a defective hemagglutinin which may show HA activity at a different pH to that used in the test. In all cases, HA was completely inhibited by specific anti-SFV serum indicating that the observed HA was virus-specific.

DISCUSSION

There is a strong correlation between the RNA phenotype and heat stability (at 50.5°) of the mutants. Most RNA^- mutants were heat stable, which accents the hypothesis that RNA^- mutants are defective in the synthesis of RNA polymerase which is probably a non-structural protein. Two RNA^- mutants, ts-5 and ts-37, were heat labile. If heat lability at 50.5° can be equated with altered configurations in structural proteins, then ts-5 and ts-37 may be double mutants. Alternatively, they may be single mutants in which the defective structural proteins interfere with viral RNA synthesis, as postulated for some of the coat protein ts mutants of poliovirus (Cooper et al, 1969).

The $RNA^{\pm}$ and most RNA^+ mutants (except ts-25, -26 and -36) were heat labile showing that the structural proteins of these mutants are different from those of wild-type virus. The mutational event which rendered some mutants unable to grow at the restrictive temperature was probably also the mutation that rendered them more heat labile at 50.5°. This was inferred from the observation that wild-type revertants of 2 heat labile mutants, ts-18 and ts-32, regained the full heat stability of wild-type virus. However, reversion in ts-33 did not restore the parental wild-type phenotype. A similar observation was reported by Burge and Pfefferkorn (1966a) for Sindbis virus ts mutants. They suggested that reversion occurred through intracistronic suppression, i.e. revertants may contain a second amino-acid substitution at a site distinct from the original mutation which allows the

protein to function at the restrictive temperature, but did not restore heat stability.

The preliminary data of heat inactivation of infectivity at the restrictive temperature suggest a different mode of inactivation to that at 50.5°. Mutant ts-36 which is relatively heat stable at 50.5°, is labile at 38.5°, and vice versa for ts-28. The significance of this is not clear, and may result from inactivation of viral RNA (Ginoza, 1968).

Of the 4 RNA^+ mutants prepared at 30° (ts-4, -21, -23, -26), only ts-23 seemed to have normal HA. Surprisingly, ts-21, which caused infected cells to adsorb gander red blood cells (see Chapter 5), was defective in HA. Assuming that both hemadsorption and hemagglutination are affected by the same protein (there is no reason to assume the contrary), then it seems that this protein is labile and lost its activity during the acetone extraction procedure.

The RNA^- mutant, ts-6, had a higher HA to PFU ratio than wild-type virus. In hemadsorption tests (see Chapter 5) this mutant induced higher levels of hemadsorption than wild-type virus. Thus, it seems that ts-6 produces a protein with enhanced hemagglutinating and hemadsorbing activities.

At the permissive temperature, significant yields of infectious virions of ts-4, -21 and -26 were produced and these had little HA activity. Since the HA activity is a function of the membrane protein (Osterrieth and Calberg-Bacq, 1966), this function cannot play a significant role in the maturation of the virions of these mutants at the permissive temperature.

CHAPTER 8

ELECTRON MICROSCOPIC STUDY OF VIRUS - INFECTED CELLS

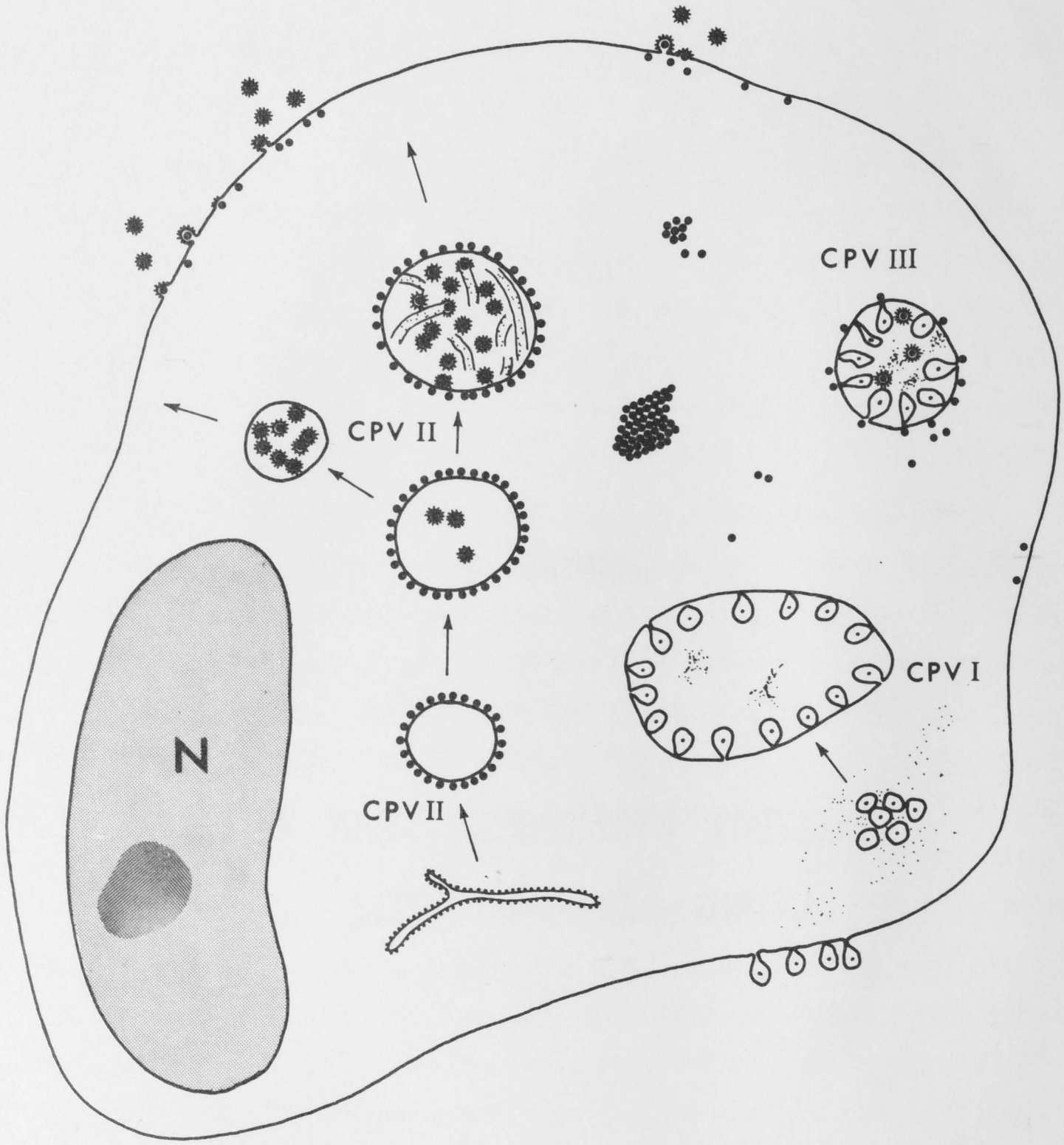


Figure 8-1. Schematic diagram of SFV infected cell.

CPV I appear to arise from spherular clusters in the cytoplasm. CPV II appear to arise from the endoplasmic reticulum and are surrounded by viral nucleocapsids. Nucleocapsids are also found in aggregates in the cytoplasm. Late in infection CPV II may contain tubules thought to be viral membrane protein and virions. CPV II may migrate to the cell surface to discharge their contents. Mature virions may also exit in transport vacuoles (CPV III). N = nucleus.

INTRODUCTION

This Chapter reports a study, in the electron microscope, of cells infected with three ts mutants of SFV. This study was aimed at correlating the morphological defects of the mutants with their physiological defects (reported in Chapters 4, 5 and 6), and elucidating the role played by virus-specific structures which were seen in infected cells.

Morphological changes produced after SFV infection of chick embryo cells ((a) Acheson and Tamm, 1967; (b) Grimley et al, 1968), HeP2 cells ((c) Erlandson et al, 1967) and mouse brain cells ((d) McGee-Russel and Gosztonyi, 1967) have been reported by several authors. Their findings, which are similar, are presented diagrammatically in figure 8-1.

The virus seems to enter the cell by phagocytosis[(b)] but it is not known how the virus particle or its components enter the cytoplasm from the phagocytic vacuoles. Two and one half hours after infection, virus particles were seen 'budding' at the cell membrane, also large vacuoles (0.6- 2 μ in diameter) with small spherules (35-100 mμ in diameter) in their lumen attached to the vacuolar wall, appeared. These vacuoles have been called 'Cytopathic Vacuoles I' or CPV I[(b)] and appear to originate from spherular clusters in the cytoplasm. In an autoradiographic study of early viral RNA synthesis[(b)], grains were concentrated near the cell membrane and in the region of CPV I, suggesting that these are the places where viral RNA is synthesised.

About 4 hours after infection unique small vacuoles (called CPV II, diameter of about 0.35 μ [b]) appeared. CPV II seemed to originate from the rough endoplasmic reticulum and they were covered with viral nucleocapsids. Grimley et al[b] suggested that CPV II and the cell membrane provide sites for the assembly of viral RNA and proteins into nucleocapsids. Nucleocapsids were seen in large numbers in the cytoplasm, some of which were 'budding' at the cell membrane.

While virus was being produced most rapidly (5-9 hours in chick cells[a] and about 12 hours in HeP2 cells[c]), CPV I were found in decreasing numbers while CPV II increased in number. Some CPV I contained degraded spherules which could have resulted from a combination with lysosomes[b]. Nucleocapsids and budding virus particles were abundant.

Late in infection cells showed cytopathic effects i.e. they rounded up, were highly vacuolated, the nucleus (nuclear membrane may be absent) was displaced to the periphery of the cell and ribosomes seemed to be more plentiful than before probably due to the smaller size of the rounded cell[a]. Nucleocapsids occurred mainly in crystalloid aggregates or around CPV II[a]. Many CPV II contained virus particles; it was suggested that they may migrate to the cell surface and release the virus particles by a process of 'reverse phagocytosis'[b]. Some CPV II had spherules attached to the inside wall (they are now called CPV III[b]), these also were thought to be transport vacuoles involved in the transport of virus particles to the exterior.

Tubules (diameter of about 40 mμ and up to 280 mμ in length) which were only present late in infection, were seen in CPV II[a]. Occasionally virus particles were attached to the ends of the tubules[c] and these tubules were suggested to be rods of viral membrane protein[a,d].

No vacuoles, spherules and tubules were observed in the nucleus, nor in the cytoplasm of uninfected cells; they are presumably virus specific.

To summarise, the suggested process of formation of a SFV particle is: a nucleocapsid approaches the cell membrane where it buds off and is released covered with an envelope.

RESULTS

Minilayers prepared from one batch of cells were infected at a multiplicity of about 30 PFU/cell with wild-type virus and 3 RNA^+ mutants; ts-15, which made both nucleocapsids and viral membrane protein but not infectious virus at 38.5°, and ts-18 and ts-21 which made only the membrane protein at 38.5° (see Chapter 5). After 14 hours incubation at 30° or 38.5° in the absence of AMD, thin sections of cells were prepared (as described in Chapter 2) for examination in the electron microscope.

Several sections of each sample were examined and representative electron micrographs of these are shown in Plates 1 to 10 and will be described in detail. It will be assumed that nucleocapsids in evaginations of cellular membranes are budding virus particles and not virus particles entering the cell.

PLATE 1

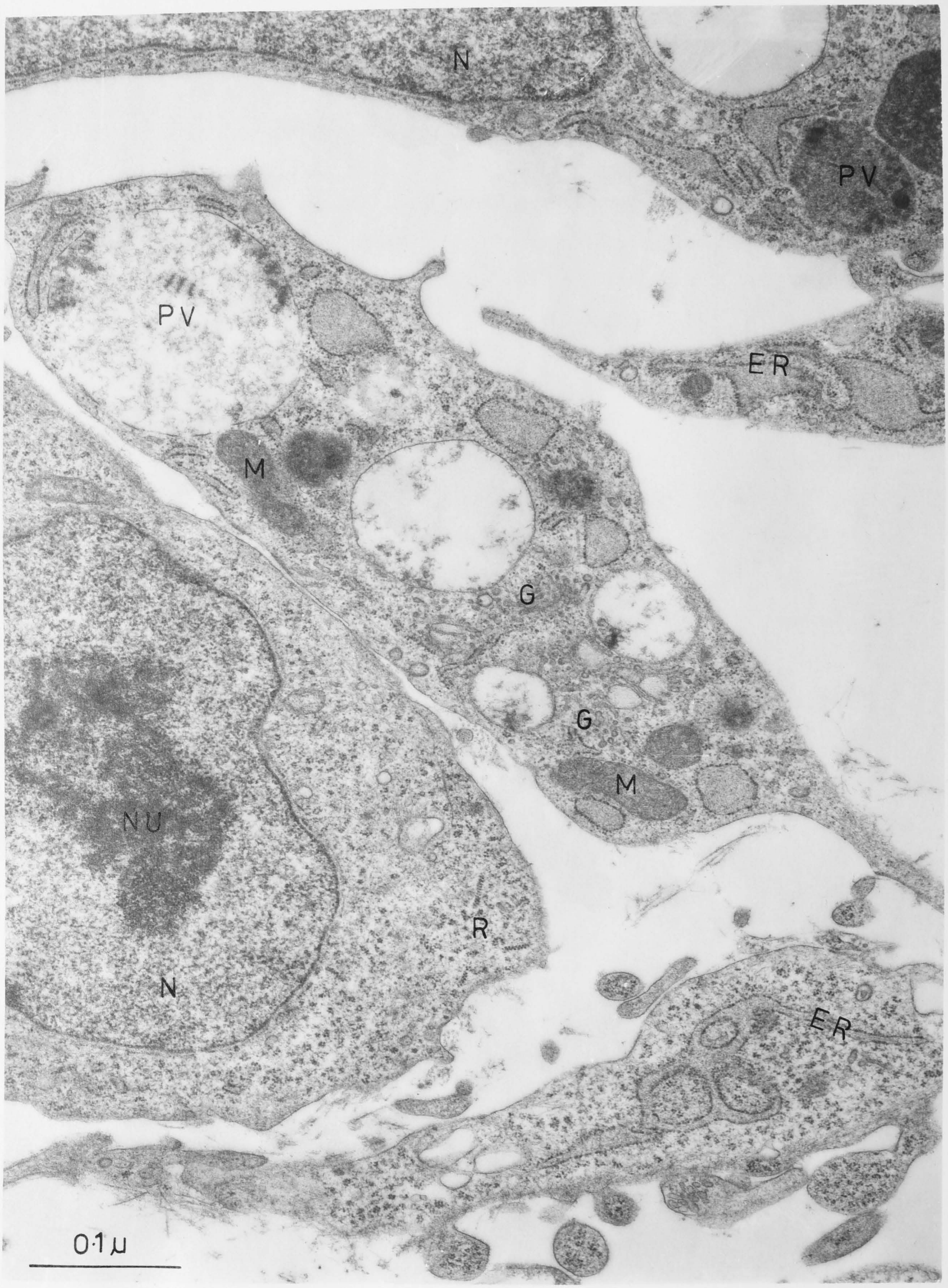


PLATE 1

UNINFECTED CHICK EMBRYO CELLS

In uninfected cells the nucleus (N) has a distinct nuclear membrane and nucleolus (NU). Ribosomes (R) are abundant and are associated with the endoplasmic reticulum (ER) or forming small aggregates in some of which the ribosomes are arranged helically. Such aggregated ribosomes are characteristic of undifferentiated cells. Other cell structures present are: mitochondria (M), golgi complex (G) and phagocytic vacuoles (PV) of various sizes. No attempts were made to identify lysosomes. The contents of the PV on the left of Plate 1 are probably materials taken from the growth medium.

Numerous fibres are present and are characteristic of chick cells grown in monolayer cultures, most of which are fibroblasts. In the lower part of Plate 1 many cellular filaments or extensions are cut in transverse section.

(magnification of Plate x 24,500)

PLATE 2

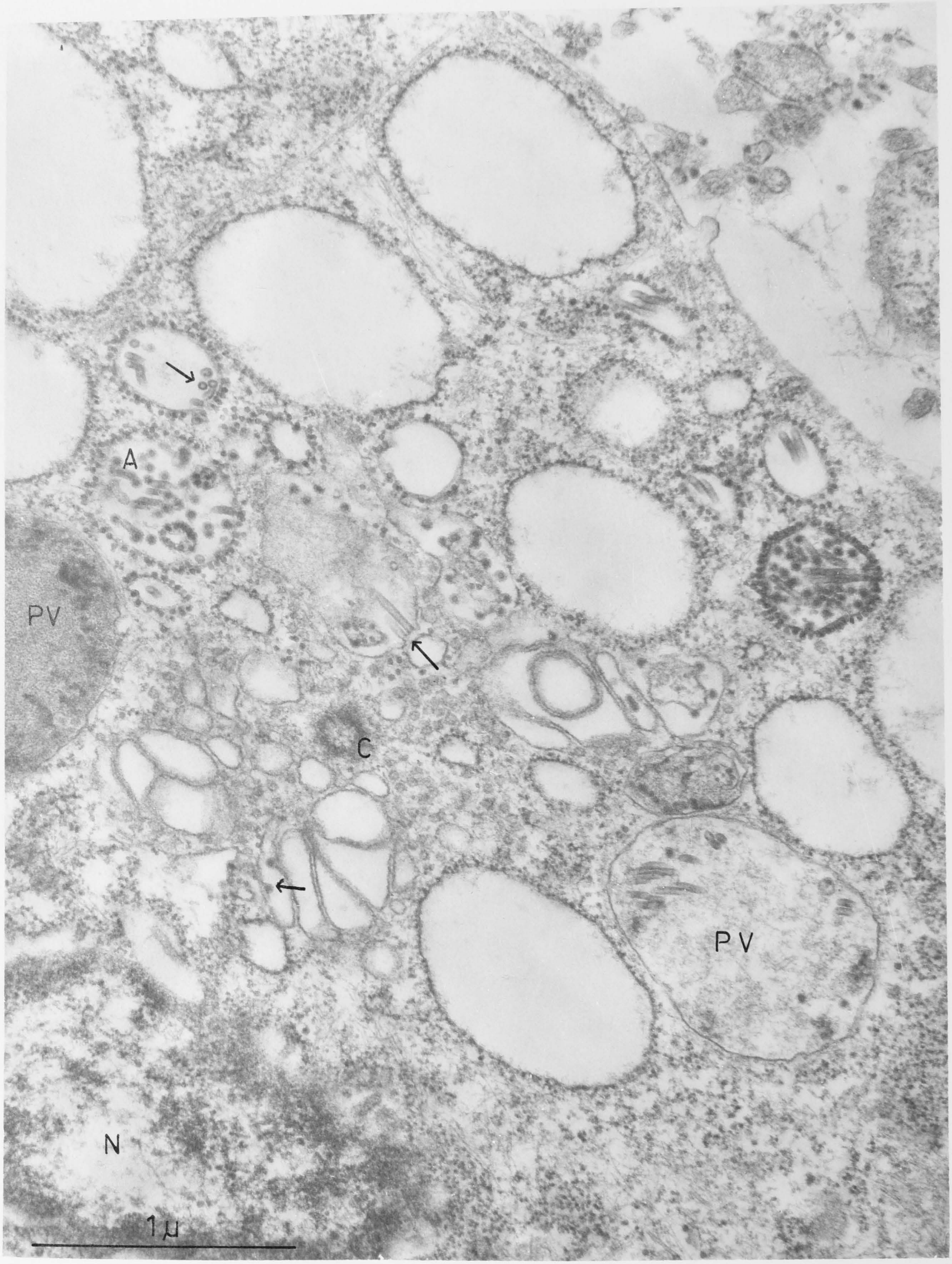


PLATE 2

CELLS INFECTED WITH WILD-TYPE SFV AT 30°
(14 hours after infection)

The cells are vacuolated and contain numerous CPV II. In the vacuole above the centriole (C) in Plate 2, two tubules are attached to the vacuolar membrane (arrow). In other vacuoles the tubules are cut in transverse section (arrow, top left). In CPV II at A, virus particles are found at the ends of tubules, one of which contained 3 nucleocapsids. These tubules may be rods of viral membrane protein.

Two virus particles seemed to be budding into a vacuole (arrow below C). In other sections (not shown) virus particles were seen budding at the cell membrane.

Spherules (although not in Plate 2) were found scattered in the cytoplasm or attached to the cell membrane. CPV I were absent and CPV III were rarely found. Cells infected at 38.3° (not illustrated) were similar in appearance to cells infected at 30°.

N = nucleus; PV = phagocytic vacuole.
(magnification of Plate x 46,000)

PLATE 3

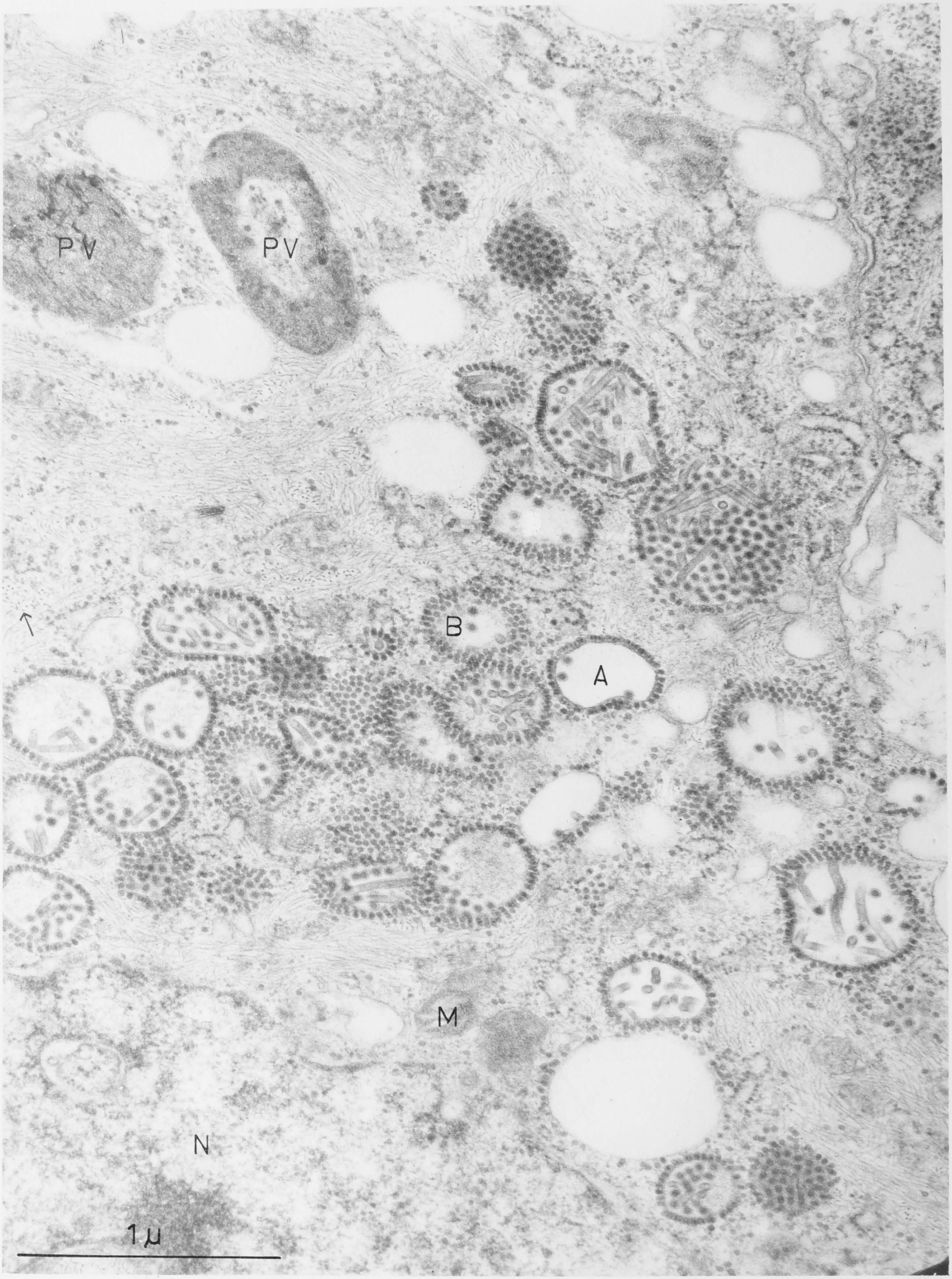


PLATE 3

CELLS INFECTED WITH *ts*-15 AT 30°
(14 hours after infection)

There are numerous CPV II, some of which were cut at a glancing angle (e.g. B in Plate 3). Most CPV II contain virus particles and tubules cut at various angles. Three budding virus particles are seen in the CPV II at A. Nucleocapsids were never found in CPV II or in the nucleus (N). Nucleocapsids are arranged in crystalline arrays in a number of aggregates.

Note the close proximity of CPV II and the cell membrane on the right. Virions in CPV II may be released to the exterior by a process of 'reverse phagocytosis'.

Fibres are abundant and are characteristic of chick embryo fibroblasts. When cut transversely the fibres are seen as black dots (arrow, left).

Spherules were present in large numbers in or on other cells.

M = mitochondria; PV = phagocytic vacuole.
(magnification of Plate x 46,000)

PLATE 4

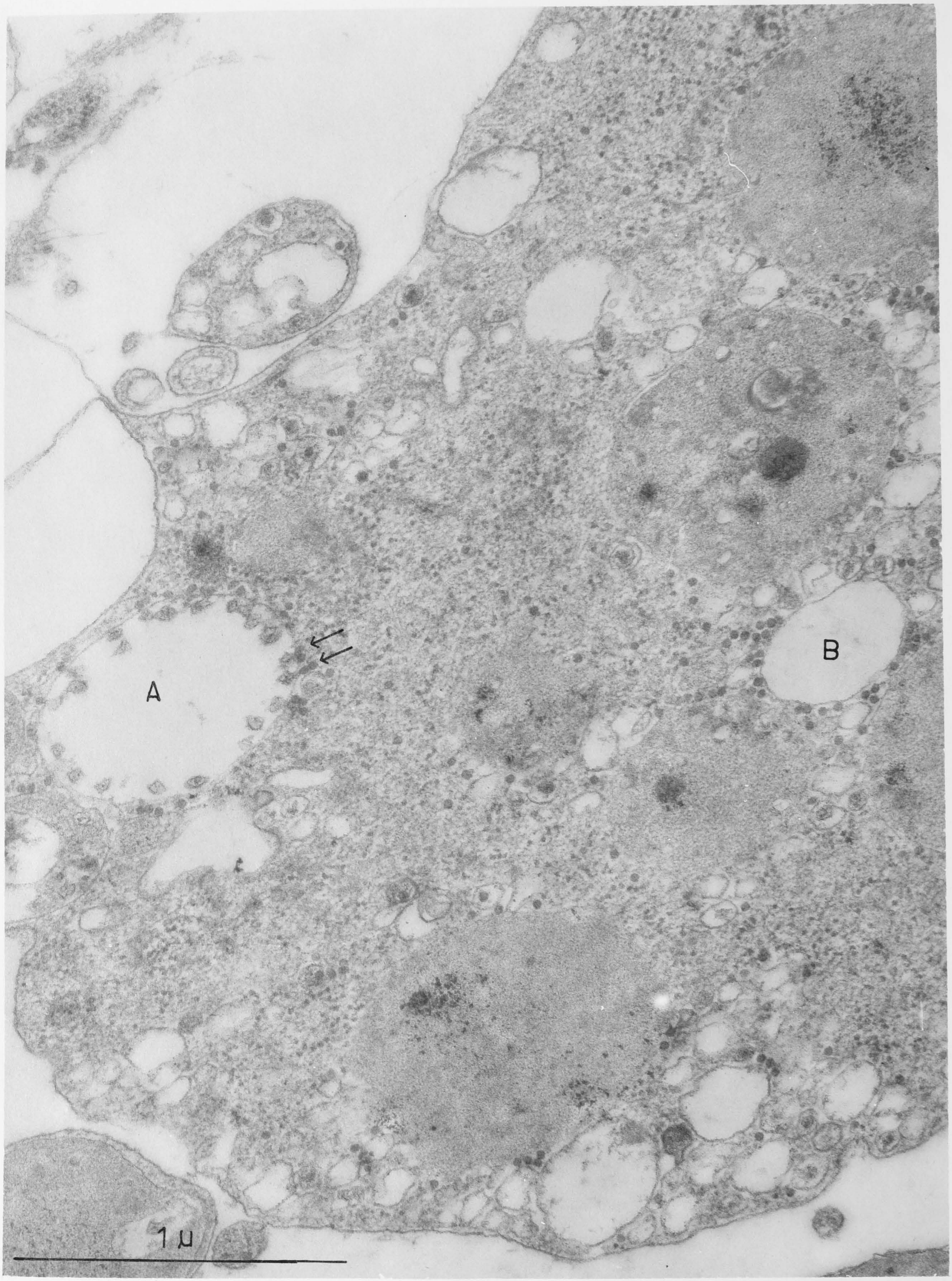


PLATE 4

CELLS INFECTED WITH ts-15 AT 38.5°
(14 hours after infection)

Nucleocapsids are scattered in the cytoplasm (which is highly vacuolated) or associated with CPV II (B) or CPV III (A) (Plate 4). Nucleocapsids associated with CPV III often lie opposite spherules (arrow). Crystalloid aggregates of nucleocapsids seen in cells infected at 30° were not detected in cells infected at 38.5°.

In other sections budding virus particles were seen but in a negligible number when compared to those found in cells infected at 30°. These budding virus particles can be attributed to leak growth.

(magnification of Plate x 58,800)

PLATE 5

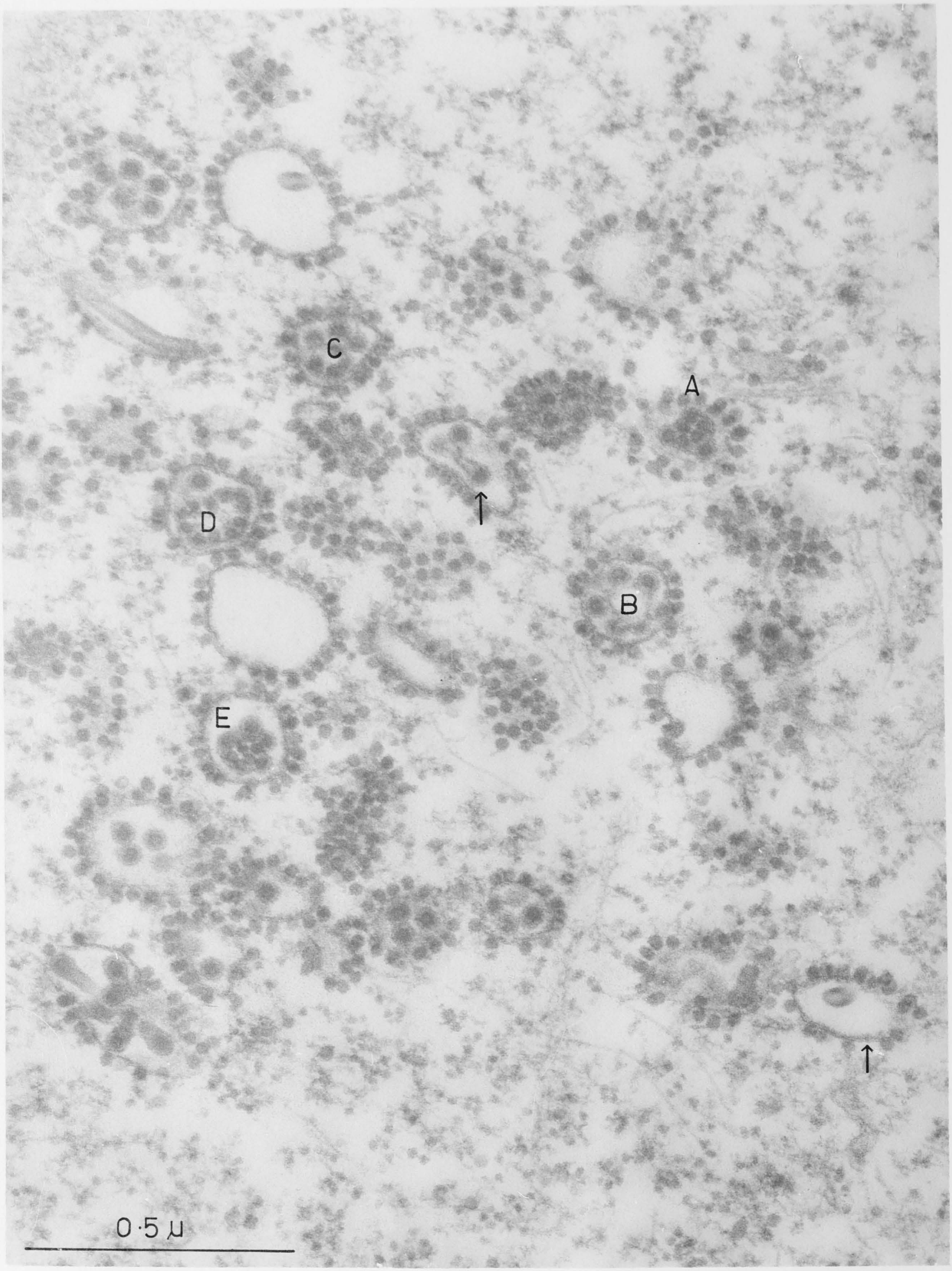


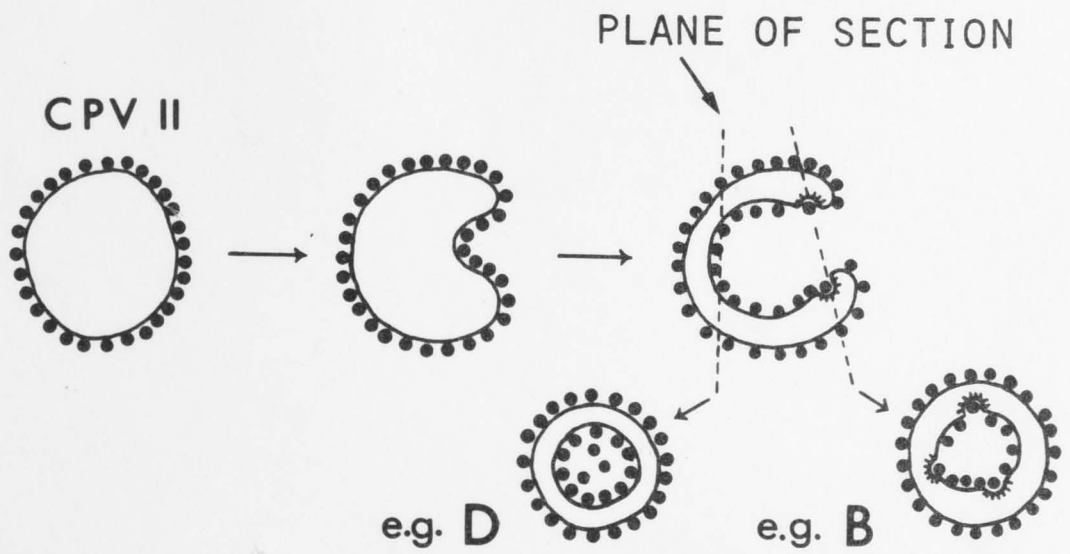
PLATE 5

CELLS INFECTED WITH ts-18 AT 30°

(14 hours after infection)

This is a picture of a typical cell late in infection (plate 5). It is characterised by the presence of nucleocapsid aggregates and numerous CPV II. The membrane of CPV II is of unit-membrane structure (arrow, bottom right). Similarly, the tubule to which a virus particle is attached (arrow, middle) also displays a unit-membrane wall.

At A, B, C, D and E, the CPV II are probably inverted so that nucleocapsids or virus particles appear to line the inside of vacuoles within the CPV II; this is represented diagramatically below:



(magnification of Plate X 95,000)

PLATE 6

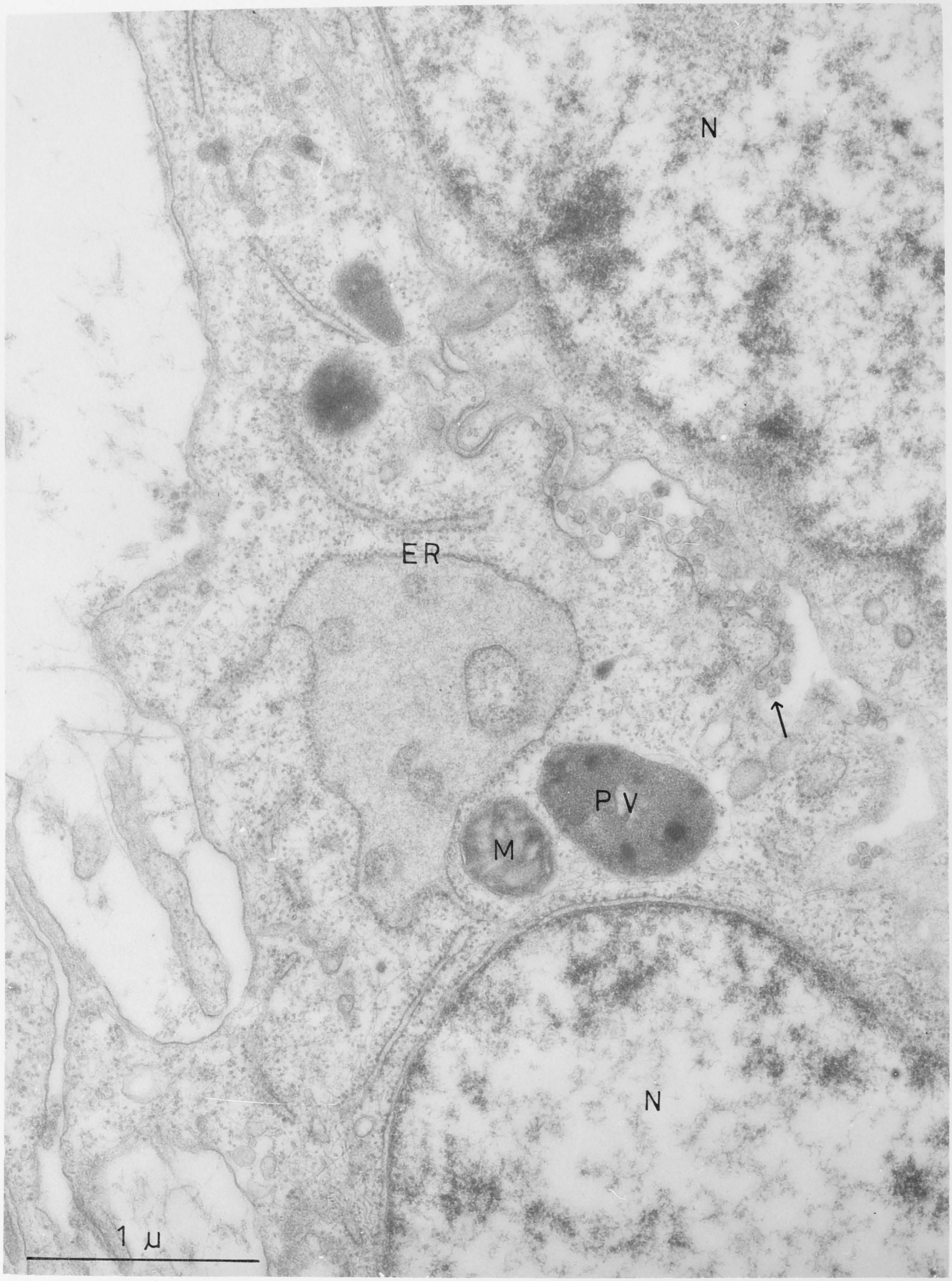


PLATE 6

CELLS INFECTED WITH ts-18 AT 38.5°
(14 hours after infection)

The cells are normal in appearance except for the presence of spherules between the 2 cells (arrow, Plate 6). Some of the spherules are attached to the cell membranes. Compare with Plate 4 - where the spherules are attached to the inner wall of the vacuolar membrane.

Nucleocapsids and virus particles were seen in other sections but in very small numbers and are probably due to leak growth.

ER = endoplasmic reticulum; M = mitochondria;
N = nucleus; PV = phagocytic vacuoles.
(magnification of Plate x 40,500)

PLATE 7

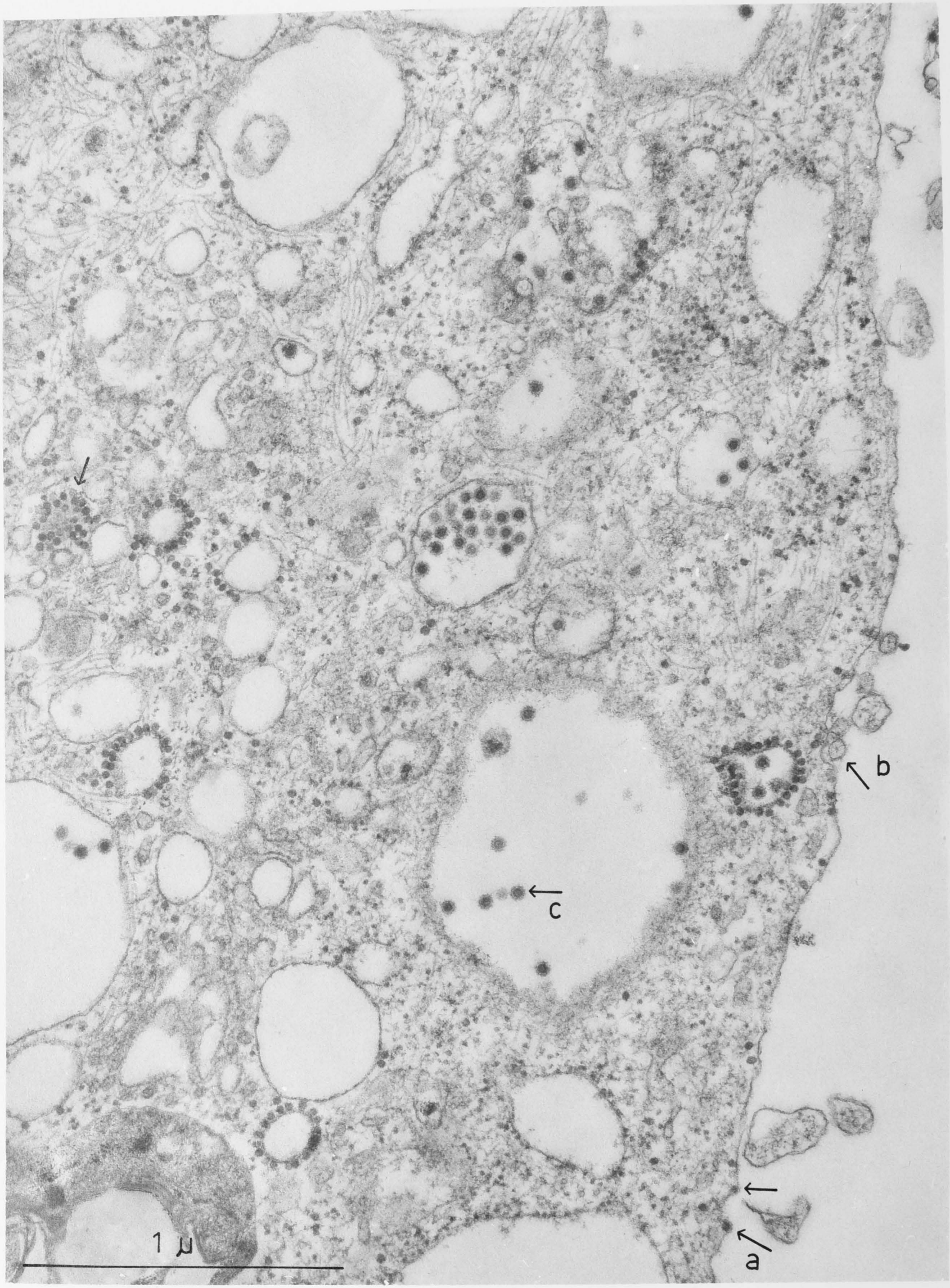


PLATE 7

CELLS INFECTED WITH ts-21 AT 30°
(14 hours after infection)

The cell is highly vacuolated and contains a few CPV II and an aggregate of nucleocapsids (arrow, left; Plate 7). Note that the nucleocapsids of many virus particles have a polygonal shape and a translucent centre with a centrally placed electron dense dot (arrow C). Nucleocapsids are also found attached to the cell side of the cell membrane and 2 virus particles in buds are seen at arrow a. Spherules are also associated with the cell membrane (arrow b). In other sections rare CPV I were seen.

(magnification of Plate x 56,000)

PLATE 8

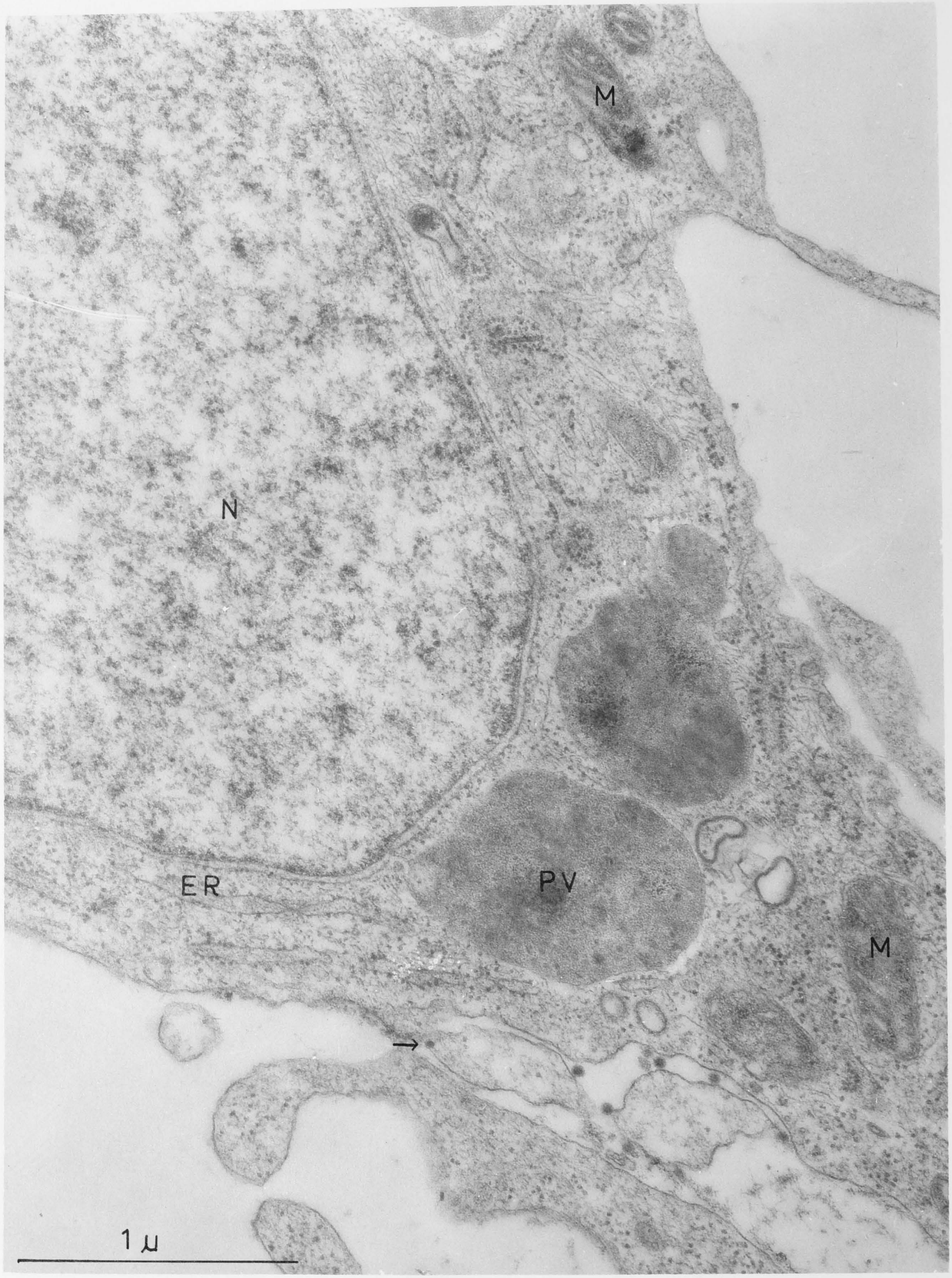


PLATE 8

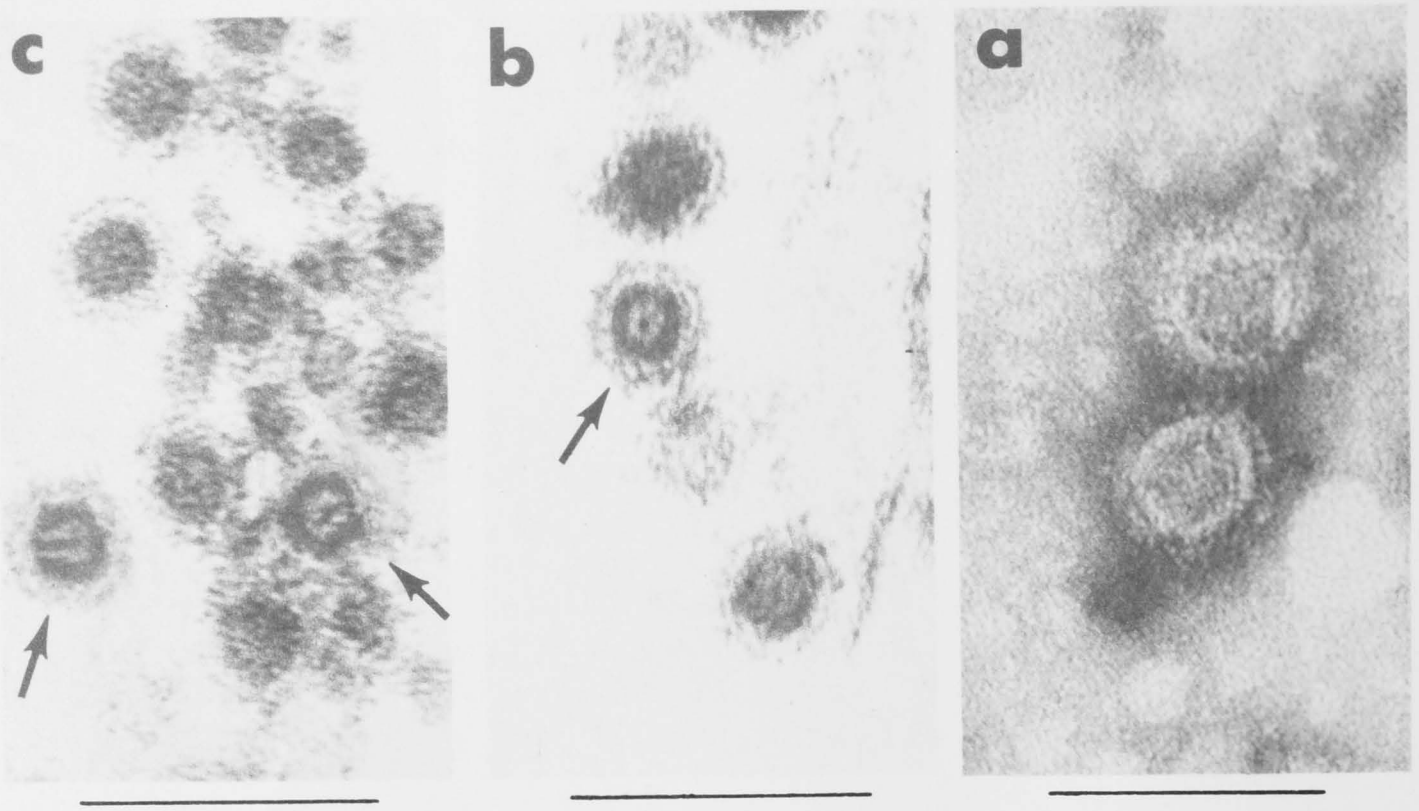
CELLS INFECTED WITH ts-21 AT 38.5°
(14 hours after infection)

The cell is normal in appearance except for the presence of a few virus particles and a budding virus particle (arrow, Plate 8). These are probably the products of leak growth.

Spherules were not detected in this or other sections.

ER = endoplasmic reticulum; M = mitochondria;
N = nucleus; PV = phagocytic vacuole.
(magnification of Plate x 49,000)

PLATE 9



scale = **1000 Å**

PLATE 9

THE MORPHOLOGY OF VIRUS PARTICLES AND NUCLEOCAPSIDS

(a) Virus particles negatively stained with sodium silicotungstate. The envelope appeared to be damaged and stain seemed to have penetrated the virus particle to reveal an internal component.

(b) Virus particles in thin section of infected cell stained with uranyl acetate and lead citrate. The spikes on the envelope are poorly stained. One nucleocapsid (arrow) has a distinct electron lucent centre which contains an electron dense body.

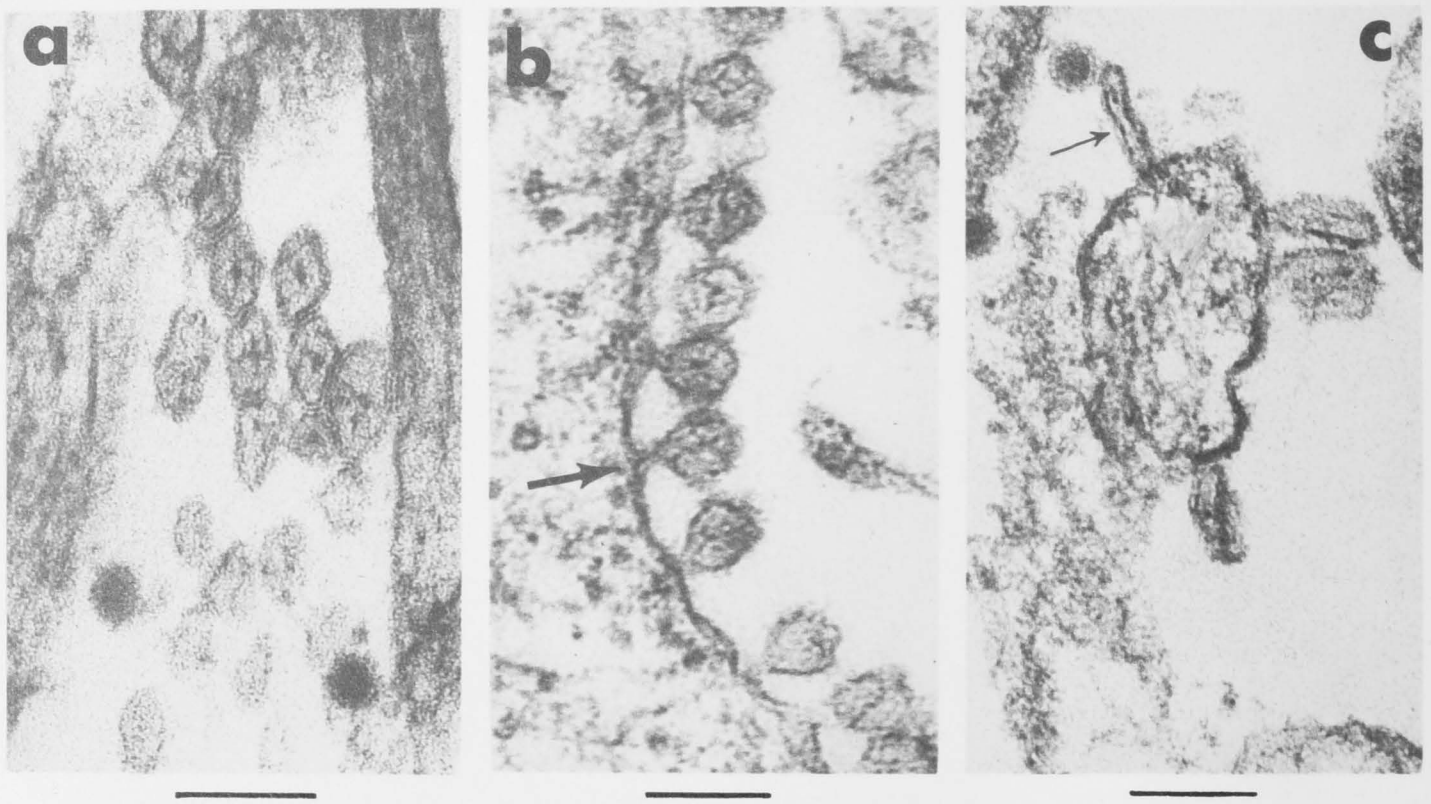
(c) Virus particles in thin section of infected cell. In two virus particles (arrows) an electron dense rod traverses the electron lucent centre of the nucleocapsid. This electron dense rod when cut transversely probably resulted in the appearance shown in (b), and is suggested to be the terminal ending of a wound nucleocapsid component (Simpson and Hauser, 1968b).

(d) and (e) Thin sections of infected cells showing virus particles budding at the cell membranes. The triple layered unit-membrane of the cell is clearly visible (arrows) and can be followed into the developing virus particles.

(f) A group of nucleocapsids in thin section of infected cell. Some of the nucleocapsids have an electron lucent centre with a centrally placed electron dense body (arrow).

(g) A large crystalloid of nucleocapsids in thin section of infected cell. The nucleocapsids are arranged in a crystalline array.

PLATE 10



scale = **1000** Å

PLATE 10

THE MORPHOLOGY OF SPHERULES AND TUBULES

(a) to (f) are selected areas from electron-micrographs of virus infected cells sampled 14 hours after infection.

Spherules range in size from 493A to 840A. They have a triple layered wall and an electron dense particle in the centre (a). The membrane of the spherule is continuous with the cell or vacuolar membrane to which it is attached (arrow in (b)). In (c) the spherule (arrow) seemed to be collapsed.

Tubules are about 330A in diameter and up to 3,600A in length. They probably originate from the membrane of certain vacuoles (d). In transverse section the wall of the tubule is triple layered (e). Virus particles are often attached to the ends of tubules and tubules may contain more than one nucleocapsid arranged in a linear order (f).

Appearance of cells infected at 30°

Cells infected at 30° by wild-type virus and the 3 mutants were similar in appearance and all resembled the infected cells reported by Acheson and Tamm (1967) and Erlandson et al (1967). The observations of these cells are summarised below:

(1) Cells were highly vacuolated and contained numerous CPV II. Both virus particles and tubules were present in CPV II. Often virus particles were attached to the ends of tubules and sometimes appeared to bud into CPV II (an appearance not reported previously).

(2) CPV I were rare. Spherules were frequently found scattered in the cytoplasm, presumably resulting from the break down of CPV I. Often spherules were attached to large areas of the cell surface. Grimley et al (1968) have also observed spherules on the cell surface, but in small numbers.

(3) Nucleocapsids occurred mostly in aggregates either around CPV II or in large crystalloids, some of which contained nucleocapsids in crystalline arrays; and

(4) There was no difference in the size or morphology of the particles of wild-type virus and the 3 mutants. The virus particles were about 410A in diameter excluding the spikes. The spikes could not be measured to any degree of accuracy because they were poorly stained. Viral nucleocapsids have a polygonal shape and were usually 5 or 6 sided and measured about 280A in diameter. The

electron lucent centre of the nucleocapsids was about 190A in diameter and the electron dense particle in it was about 48A in diameter.

(5) CPV I, CPV II, CPV III, spherules and tubules were never observed in uninfected cells and these are presumably virus specific.

Appearance of cells infected at 38.5°

At 38.5° only the cells infected with wild-type virus resembled the cells infected at 30°. Cells infected at 38.5° with (i) ts-15 were vacuolated and contained nucleocapsids (but not in massive aggregates that were present in cells infected at 30°) and a few CPV III; (ii) ts-18 or ts-21 contained very few nucleocapsids, which represented a negligible fraction of the number at 30°, and these were scattered singly in the cytoplasm. Few spherules were found in cells infected with ts-15 or ts-18 and spherules were not detected in cells infected with ts-21.

In a few cells infected with the mutants at 38.5°, some budding as well as mature virus particles were seen. These and the few nucleocapsids seen in cells infected with ts-18 and ts-21 at 38.5° are probably produced by leak growth of the mutants.

DISCUSSION

The results of my electron microscopic study of cells infected with wild-type SFV or with ts mutants are in agreement with published reports.[a,b,c,d] A comparison of electron microscopic studies of cells infected with SFV and with other arboviruses has been presented in detail by Erlandson et al (1967) and will not be discussed here.

At the restrictive temperature ts-15 made nucleocapsids but not complete virus particles whereas both ts-18 and ts-21 appeared defective in the production of nucleocapsids, as investigated with the electron microscope. This finding is in complete agreement with the results obtained with physiological tests (see Chapter 5). In these tests it was concluded that at the restrictive temperature all the 3 mutants made viral membrane protein but only ts-15 made nucleocapsids. Morphologically the cellular membrane and the viral envelope are similar in structure except for the presence of spikes in the latter. In the maturation of the virus particle, spikes were seen only on budding virus particles. Thus it was not possible, in the present study, to identify in cells infected with ts-15, -18 and -21 at 38.5°, cellular membranes which were altered biochemically by the insertion of viral membrane protein in them (suggested by hemadsorption tests, Chapter 5).

One difference between the cells infected with mutants at 38.5° and at 30° is the reduced number of (with ts-15 and ts-18) or the absence of (with ts-21)

spherules at 38.5°. Acheson and Tamm (1967) suggested that spherules could be abnormal forms of viral envelope, or could be 'incomplete' virus particles, or phagocytosed remains of virus particles. Grimley et al (1968) suggested that spherules may be associated with viral RNA synthesis. The 3 SFV mutants studied made viral RNA at 38.5° (see Chapter 4) but made few or no spherules under similar conditions. This suggests that spherules are not associated with viral RNA synthesis; their nature and function remain to be elucidated.

Tubules were not detected in cells infected with mutants at 38.5°. It seems that tubule production is associated with the ability of the virus to complete the normal growth cycle. Hiashi et al (1967) saw such tubules, some of which were attached to the cell membrane, in cells infected with Chikungunya virus but did not comment on their significance. McGee-Russel and Gosztonyi (1967) and Acheson and Tamm (1967) suggested that tubules were viral membrane protein. My observations that (i) tubules were attached to the membrane of CPV II, (ii) virus particles were found at the ends of tubules, and (iii) some tubules contained more than one nucleocapsid, led to the interpretation that a nucleocapsid budding into CPV II was not 'nipped off' to form a virus particle; instead, the viral membrane protein continues to be made, resulting in the formation of a tubule. If other nucleocapsids happen to bud at the same spot then the resulting tubule will contain more than one nucleocapsid. I therefore conclude that tubules are viral membrane protein resulting from abnormal budding of virus particles.

Nucleocapsids cut in thin sections often are polygonal in shape and are usually 5 or 6 sided. They often occur in large crystalloids in some of which nucleocapsids are arranged in a crystalline array. Acheson and Tamm (1967) and Simpson and Hauser (1968a) suggested that nucleocapsids have cubic symmetry. The nucleocapsids and virus particles of wild-type SFV and the mutants were similar in size and morphology. The nature and function of the tiny electron dense body located in the centre of the nucleocapsid are not known.

CHAPTER 9

GENETIC STUDIES WITH *ts* MUTANTS : COMPLEMENTATION AND RECOMBINATION

INTRODUCTION

Burge and Pfefferkorn (1966b) found that Sindbis virus ts mutants complemented with relatively high frequencies. Using complementation tests they separated 11 mutants into 5 non-overlapping groups, which for the RNA^+ mutants, were the same as the grouping based on their functional defects (Burge and Pfefferkorn, 1968). Recombination was not demonstrable between Sindbis ts mutants although the system was sensitive, i.e. any recombination of the order of 10^{-4} could be detected (Burge and Pfefferkorn, 1966b). Sambrook (1965) reported both recombination and complementation between certain pairs of SFV ts mutants. However, his preliminary results did not allow the mutants to be placed in any definite groups.

As the physiological defects of a large number of SFV ts mutants were known, complementation and recombination tests were attempted to locate and map the genetic lesions of these mutants. Particular emphasis was placed on complementation between RNA^- mutants because of a possibility that 2 viral enzyme functions may be involved in viral RNA replication (see Chapters 4 and 6).

COMPLEMENTATION

Methods

In single infections, minilayers were infected with about 30 PFU per cell. In double infections, each cell was infected with about 15 PFU of each of 2 mutants. After overnight incubation at 4^{o} in the presence of AMD, the cells were washed and incubated at 38.5^{o} in the presence of AMD. After 4 hours incubation at 38.5^{o}, the medium was replaced with fresh prewarmed medium containing AMD. This is to remove virus which could not be washed off the cells at the end of the adsorption period and which had subsequently eluted into the growth medium during the early phase of the growth cycle. Burge and Pfefferkorn (1966b) reported that it was essential to remove the eluted virus which could partially or completely mask the occurrence of complementation between ts mutants of Sindbis virus. After 10 hours incubation at 38.5^{o}, infected cells were frozen, thawed and assayed for infectivity at 30^{o}.

Each experiment was done in duplicate as was the plaque assay of yields. The difference between duplicate yields or plaque assays rarely exceeded 2 fold. Thus if the yield of doubly infected cells exceeded the sum of the yields of cells infected with each of the 2 parental mutants (e.g. $\frac{\text{Yield AxB}}{\text{Yield A + Yield B}}$) by 2 fold or greater (called 'Complementation level'), positive complementation is said to have occurred. Complementation levels of 1 or less than 1 probably result from absence of complementation or interference of viral yield, respectively.

Figure 9-1.

Complementation between RNA$^-$ mutants.

	3										
3	10	5									
5	12 1.1	0.7	6								
6	25 2.1	5 1.9	2	7							
7	16 1.1	1 0.2	2 0.3	4	9						
9	27 1.2	58 4.6	56 4.0	10 0.6	12	10					
10	38 2.4	57 8.5	47 5.8	5.3 5.3	12 0.7	16	11				
11	40 2.9	39 8.3	25 4.2	15 1.9	19 1.2	21 2.1	4	16			
16	8 0.7	4 1.5	46 11.5	8 1.3	8 0.6	7 0.9	8 1.3	2	19		
19	17 1.1	3 0.5	22 2.8	16 1.6	22 1.2	3 0.3	8 0.8	12 1.5	6	37	
37	53 1.8	28 1.4	44 2.0	24 1.0	31 1.0	18 0.7	31 1.3	22 1.0	10 0.4	20	38
38	136 1.4	103 1.2	209 2.4	147 1.6	17 0.2	22 0.3	60 0.6	86 1.0	22 0.3	102 1.0	86

The top figure in each square is the yield (PFU per ml x 10^2) of doubly infected cells and the bottom figure is the Complementation Level. Pairs showing significant complementation are underlined.

Figure 9-2.

a Complementation between RNA^- and RNA^+ mutants.

		6	11	8	28
(RNA^-)	6	0.6			
(RNA^-)	11	6 4.3	0.2		
(RNA^+)	8	5 3.6	1.39 0.6	0.8	
(RNA^+)	28	23 4.5	12 2.0	8 1.6	4.5

b Complementation between RNA^+ mutants.

	8	21	25	26	30	32	36
8	0.2	21					
21	0.5 1.1	0.3	25				
25	23 13.5	11.5 6.4	1.5	26			
26	6 1.4	14 3.1	11 1.9	4.2	30		
30	3.2 0.5	8.5 1.3	12 1.6	15.2 1.5	6	32	
32	5 3.1	8.5 5.0	10.5 3.6	6 1.1	8.2 1.1	1.4	36
36	9 5.3	1.0 5.6	11 3.7	10 1.8	16 2.1	4 1.4	1.5

The top figure in each square is the yield (PFU per ml x 10^4) of doubly infected cells and the bottom figure is the Complementation level. Pairs showing significant complementation are underlined.

Results

All RNA^- mutants, and random or selected crosses between RNA^- and RNA^+, and between RNA^+ mutants, were tested. All the tests were done at least twice and the results of 3 experiments are shown in figures 9-1 and 9-2. Complementation, although demonstrable, was rather inefficient (Complementation levels from 2-14) and not reproducible. Only 2 mutants, ts-6 and ts-11 (both RNA^-) complemented consistently. The absence or low levels of complementation, especially between RNA^- and RNA^+ mutants which must belong to 2 different cistrons, could result from the experimental technique employed. A number of factors which could affect complementation were investigated. These were:

(a) Effect of AMD

A host mediated function may be required for genetic interactions between the ts mutants and its production may be suppressed by AMD. A number of experiments were designed in which:

(i) the period of overnight incubation at 4^o was shortened;

(ii) the concentration of AMD used was decreased from 1 µg/ml to 0.1 µg/ml; or

(iii) AMD was omitted altogether.

However, no increase in Complementation level was obtained under the above conditions when compared with the standard infection technique.

(b) Competition by mutants for replicating sites

In the routine method cells were infected with one mutant at a time. Although the time interval between doubly infecting cells with 2 mutants was short (1 to 10 minutes), there is a possibility that the first infecting mutant may successfully compete for accessible replicating sites or for some finite resources of the cell (e.g. adsorption capacity for virus), leaving less accessible replicating sites for the other mutant. Consequently absence of complementation or interference of total viral yield could result. This problem could be overcome by: (i) premixing the 2 mutant stocks before infecting the cells, or (ii) infecting cells with lower multiplicities of virus.

Neither of these procedures enhanced complementation.

(c) Enhancement of Complementation level

Infected cells were preincubated at the permissive temperature for 45 minutes to allow for some viral replication before the incubation at 38.3^{o}. However, this did not produce any noticeable increase in the Complementation levels.

and

(d) Complementation tests in BHK 21 cells

Baby hamster kidney (BHK 21) cells support SFV growth as efficiently as primary chick embryo cells and possess the advantageous properties of being a homogeneous cell line and contain less endo-ribonuclease than chick cells (E. M. Martin, personal communication).

Table 9-1. Results of a recombination experiment involving RNA^- and RNA^+ mutants[1]

Cross	TITRATIONS				% of[2] 'wild-type' in yield
	38.5°		30°		
	Dilution	Plaque count	Dilution	Plaque count	
6 x 6 (RNA^-)	10^{-3}	2, 5 4, 3	10^{-6}	27, 20 17, 19	0.01 0.02
8 x 8 (RNA^+)	10^{-3}	0, 2 0, 1	10^{-5}	124, 132 101, 84	<0.01 <0.01
28 x 28 (RNA^+)	10^{-3}	8, 7 10, 18	10^{-5}	69, 92 81, 61	0.09 0.02
8 x 6	10^{-3}	106, 179 40, 25	10^{-7}	8, 5 5, 9	0.22 0.05
28 x 6	10^{-3}	9, 10 8, 7	10^{-5}	65, 92 90, 122	0.12 0.07
28 x 8	10^{-3}	5, 3 12, 5	10^{-5}	44, 40 17, 13	0.10 0.57

[1] Each cross or titration was done in duplicate

[2] $\frac{\text{Titer } 38.5°}{\text{Titer } 30°} \times 100$

However, the Complementation levels obtained for complementation tests carried out in BHK 21 cells were not different from those obtained with chick cells.

The results of the above 4 complementation tests were similar to that shown in figure 9-2a and are not shown.

RECOMBINATION

Methods

Cells were infected overnight at 4^{o} in the presence of AMD as in complementation tests. The next morning the cells were washed and incubated at the permissive temperature (28^{o} to 30^{o}) in the presence of AMD. Viral yields from the fourth to the tenth hour of incubation (as in complementation) at 28^{o} or 30^{o} were assayed both at 30^{o}, to measure the total yield (mutants plus wild-type recombinants), and at 38.5^{o} to measure the wild-type recombinant yield only.

Results

In no case did 'wild-type' virus in mixed infections occur in yields greater than 0.6 per cent of the total yield. In most tests less than 0.1 per cent of 'wild-type' virus was detected in mixed infections. Yields from duplicate cultures were variable (Table 9-1). Plaques formed at 38.3^{o} were seldom wild-type in character. They probably result from leak growth.

Sambrook (1965) reported that recombination was more readily demonstrable when cells were incubated at

38.3° (as in complementation) instead of 28°. A number of tests were performed in which the experimental conditions were altered, such as, infecting cells at 38.3°, reducing the concentration of or omitting AMD from the growth medium, premixing the 2 mutants before infecting the cells, using low multiplicities of infection, and doing the test in BHK 21 cells. None of these tests produced any detectable (0.1 per cent or more of total yield) virus which formed wild-type plaques when assayed at 38.5°.

DISCUSSION

The results of both complementation and recombination tests were disappointing. Sambrook's (1965) complementation results could not be reproduced. He incubated infected cells for 18 hours (10 hours in the present experiments) at the restrictive temperature and this could have resulted in heat inactivation of virus produced by cells infected with a single mutant (see Chapter 7). The virus produced by doubly infected cells may be phenotypically mixed and heat stable (Burge and Pfefferkorn, 1966b), resulting in apparently higher Complementation levels.

Complementation occurred relatively efficiently between ts mutants of Sindbis virus, with Complementation levels from 5 to 200. But, the absolute efficiency was low, being only 1-3 per cent of wild-type virus yield under the same conditions. It is surprising that complementation occurred so inefficiently between ts mutants of SFV. Both SFV and Sindbis virus are similar in many respects and their mutants possessed similar morphogenetic defects in growth (Pfefferkorn and Burge, 1968; Tan et al, 1969), and were expected to show similar genetic interactions.

Sambrook (1965) claimed to have demonstrated recombination between certain pairs of SFV ts mutants when infection was carried out at 38.3° but not at 28°. The significance of this observation compared with the inability to demonstrate recombination in the present study is not known.

A possible explanation for the absence of recombination and the inefficient complementation is that most of the mutants are double mutants. However, as described in Chapters 4, 5 and 6, a number of the mutants appeared to be defective in one function, only affecting either the synthesis of viral RNA polymerase, or one of the two structural proteins, or a maturation function. It seems unlikely that these mutants, which were used for the genetic experiments, are double mutants.

CHAPTER 10

GENERAL DISCUSSION

AND

CONCLUSIONS

GENERAL DISCUSSION

In the past markers like virulence, plaque morphology and physiological changes in coat proteins and serological properties, were used in the study of animal virus physiology and genetics. The complex nature of these markers, which were not well understood, made interpretation of results difficult. Despite the fairly large amount of effort devoted to animal virus genetics, little was achieved in the way of genetic mapping of an animal virus. A different approach to the study of viral physiology and genetics was reported by Epstein et al (1963), using conditional lethal mutants. The advantage of such mutants is that they arise from point mutation and can, in principle, be found in every essential cistron of the virus. Studies with such mutants have yielded a genetic map of the bacteriophage T4 and a characterization of many of the gene functions (Epstein et al, 1963). Fenner and Sambrook (1964) and Fenner (1965) pointed out the potential usefulness of temperature-sensitive mutants (the only class of conditional lethal mutants readily obtainable with animal viruses) in the study of the physiology and genetics of animal viruses. Temperature-sensitive mutants have now been isolated from and provided valuable information on the mechanism of replication and genetics of a number of animal viruses (Fenner 1969).

Semliki Forest virus was chosen by Sambrook (1965) to study the physiology and genetics of an animal ribovirus. He obtained, and studied in some detail,

Table 10-1. SUMMARY OF PHYSIOLOGICAL CHARACTERIZATION OF SFV *ts* MUTANTS[a]

Mutagen	*ts* mutant	RNA phenotype	Cut-off tempt. (°C)[b] Yield -AMD	Yield +AMD	Plaque formation	Critical period of growth cycle at 38.5°	Heat stability of infectivity at 50.5°	Antigen production at 38.5°	Nucleocapsid production at 38.5°	Hemadsorption[c] at 38.5°
5-FU	3	-	36	36	38	Early	+	-	ND	ND
5-FU	6	-	38	38	36	Early	+	-	-	-
5-FU	7	-	36	38	36	Early	+	-		
5-FU	9	-	38	38	38	Early	+	-		
5-FU	10	-	37	38	38	Early	+	-		
HA	11	-	38	38	38	Early	+	-	ND	ND
HA	16	-	37	38	38	Early	+	-		
HA	19	-	37	38	38	Early	+	-		
NTG	38	-	ND	ND	38	Early	+	-		
5-FU	1	-	34	36	36	Late	+	-	ND	ND
5-FU	5	-	34	37	34	Late	-	-	ND	ND
NTG	37	-	ND	ND	36	Late	-	-	ND	ND
5-FU	2	±	38	36	36	Late	-	+	+	ND
5-FU	4	+	36	38	ND	Late	-	+	+	-
HA	12	+	38	37	38	Late	-	+	+	+
HA	15	+	37	38	ND	Late	-	+	+	+
HA	18	+	36	36	38	Late	-	+	-	+
HA	21	+	38	37	38	Late	-	-	-	+
NTG	23	+			38	Late	-	+	-	-
NTG	28	+			38	Late	-	+	+	+
NTG	31	+	ND	ND	38	Late	-	+	-	+
NTG	32	+			38	Late	-	+	-	+
HA	33	+			38	Late	-	+	-	+
NTG	35	+			38	Late	-	+	-	+
NTG	25	+	ND	ND	38	Late	+	+	+	+
NTG	26	+	ND	ND	38	Late	+	+	+	-
NTG	36	+	ND	ND	38	Late	+	+	ND	ND
5-FU	8	+	36	36	38	Late	-	+		
HA	13	+	38	37	ND	Late	-	+		
HA	14	+	38	38	ND	Late	-	+		
HA	17	+	38	38	36	Late	-	+		
5-FU	20	+	36	37	36	Late	-	+		
HA	22	+			ND	Late	-	+	ND	ND
NTG	24	+			ND	Late	-	+		
NTG	27	+	ND	ND	38	Late	-	+		
NTG	29	+			38	Late	-	+		
NTG	30	+			38	Late	-	+		
HA	34	+			ND	Late	-	+		

[a] Abbreviations used are:- - = defective; + = not defective or wild-type response; AMD = actinomycin D; 5-FU = 5-Fluorouracil; HA = hydroxylamine; NTG = nitrosoguanidine; ND = not determined.

[b] This is the temperature at which 99% or more inhibition of the normal yield (in a single growth cycle), or plaque formation under agar at 28°, occurred.

[c] A measure of viral membrane formed on the surface of cells.

9 ts mutants. A further suite of 29 ts mutants were isolated and these together with Sambrook's mutants, were studied in detail for this thesis. Discussion of the temperature-sensitive defects of these mutants have been presented in the preceding chapters. In the following discussion the experimental results are collated and discussed with particular reference to the mechanism of replication and genetics of encephaloviruses.

Characterization of SFV ts mutants

A summary of the results of physiological characterization is presented in Table 10-1. The mutants were placed in 3 groups based on their ability to synthesise viral RNA at the restrictive temperature: (i) 12 RNA^- mutants which synthesised less than 3 per cent of the wild-type virus yield of RNA; (ii) one $RNA^{\pm}$ mutant synthesising 5 per cent RNA; and (iii) 25 RNA^+ mutants synthesising from 12 per cent to 90 per cent RNA. This grouping is in agreement with the grouping of the mutants based on other physiological tests.

The ts defects of RNA^- mutants

These mutants can be placed in 3 groups (Table 10-2). Group A mutants are blocked in an early event of the growth cycle which is required for viral RNA synthesis. The results of temperature shift experiments (Chapter 5) indicate that the initiation of the synthesis of viral RNA polymerase is blocked at the restrictive temperature. Once synthesised at the permissive temperature, this enzyme was functional and stable at the restrictive

TABLE 10-2. FUNCTIONAL DEFECTS OF SFV RNA⁻ MUTANTS

Group	ts mutants	Critical period of growth cycle at 38·5°	Heat stability at 50·5°	Presumed defect at 38·5°
A	3,6,7,9,10,11 16,19,38	Early	Stable	Synthesis of RNA polymerase
B	1	Late	Stable	Synthesis(?) of RNA polymerase and another virus coded function(?)
C	5,37	Late	Labile	

TABLE 10-3. FUNCTIONAL DEFECTS OF SFV RNA⁺ MUTANTS

Group	ts mutants	Nucleocapsid production at 38·5°	Hemadsorption at 38·5°	Heat stability at 50·5°	Presumed defect at 38·5°
D	18,21,31 32,33,35	-	+	-	Production of nucleocapsids
E	4	+	-	-	Production of membrane protein
	26	+	-	+	
F	12,15,28	+	+	-	Maturation of virion
	25	+	+	+	
G	23	-	-	-	Double mutant?

* + = not defective or heat stable; - = defective or heat labile.

temperature. Martin (1969) studying the RNA polymerase of 2 RNA^- mutants (ts-3 and ts-6), arrived at a similar conclusion. All group A mutants have heat stable virions which presumably contain normal (wild-type) structural proteins.

Mutant ts-1 in group B is probably a double mutant with a defect in RNA synthesis and a second defect in the initiation of synthesis or maturation of structural proteins at 38.5^o. The structural proteins once incorporated into virions at 28^o were heat stable and presumably are normal in configuration.

The 2 group C mutants also have a defect expressed subsequent to viral RNA synthesis. The virions of these mutants are significantly more heat labile than wild-type virus. Presumably the structural proteins of these mutants are altered in configuration. Mutants ts-5 and ts-37 may be double mutants. Alternatively, they may be single mutants in which the defective structural proteins interfere with viral RNA synthesis.

None of the RNA^- mutants was able to inhibit host cell RNA synthesis at the restrictive temperature; this seems to be a function of progeny viral RNA.

The ts defects of RNA^+ mutants

More was learnt about viral functions from the study of $RNA^{\pm}$ and RNA^+ mutants. The $RNA^{\pm}$ mutant behaved like RNA^+ mutants and will not be discussed separately. All these mutants made the same species of RNA as wild-type virus at the restrictive temperature but in different proportions. A number of mutants (e.g. ts-2 and ts-15)

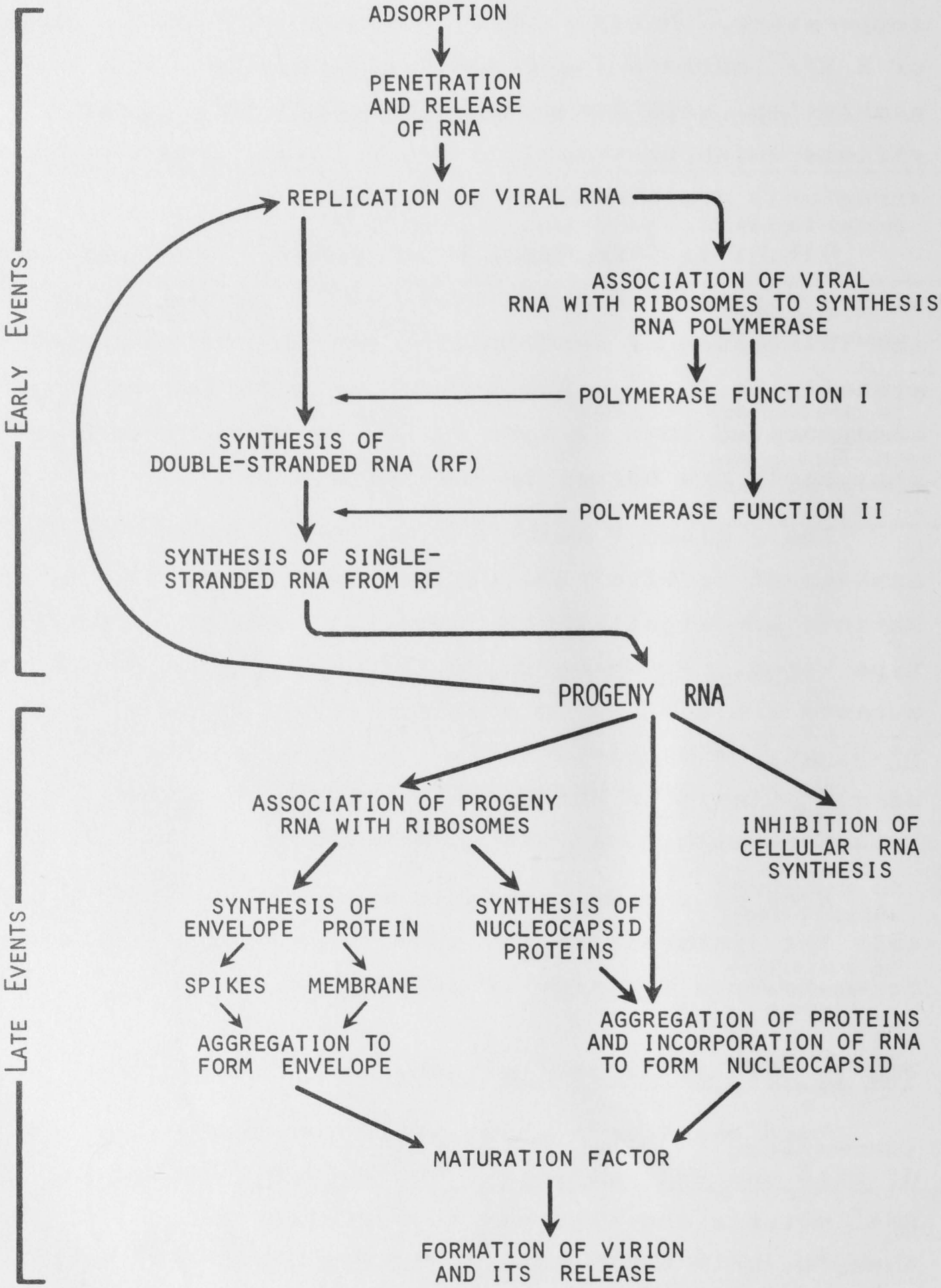


FIGURE 10-1. SCHEMATIC SUMMARY OF EVENTS OCCURRING DURING SFV REPLICATION IN A CHICK EMBRYO CELL.

made relatively large amounts of double-stranded RNA. Perhaps they are defective in an enzyme function required for the synthesis of single-stranded RNA from the double-stranded template.

The defects of RNA^+ mutants are indicated in Table 10-3. The formation of virions requires 3 major steps: (i) the formation of nucleocapsids; (ii) the formation of envelope components; and (iii) the assembly of nucleocapsids and envelopes to form mature virions. Accordingly, RNA^+ mutants can be placed in 4 groups: (D) mutants defective in the production of nucleocapsids, (E) mutants defective in the production of envelope (membrane) protein, (F) mutants which make both structural proteins but not complete virions, and (G) a suspected double mutant defective in the production of both structural proteins. The structural proteins once incorporated into virions at the permissive temperature may be heat stable at 50.5^o (configuration of proteins normal) or heat labile (configuration of proteins altered) and provide a basis for subdividing 2 of the groups (E and F).

From the study of the defects of the mutants, the scheme of SFV replication presented in figure 10-1 is proposed. Viral structural proteins may also be synthesised by the input viral RNA as well as by progeny RNA, but this is not known. This proposed scheme of viral replication is similar to those obtained with studies on the wild-type viruses of Semliki Forest virus (Friedman et al, 1966; Sonnabend et al, 1967; Acheson and Tamm, 1967), Venezuelan Equine Encephalitis virus (Zebovitz and Brown, 1967), and Western Equine

Encephalomyelitis virus (Sreevalsan and Allen, 1968; Sreevalsan et al, 1968). A similar scheme of replication is suggested by the studies on ts mutants of Sindbis virus (Pfefferkorn and Burge, 1969). Viral, double-stranded and interjacent RNAs have been extracted from cells infected with these viruses and also have been shown to have properties similar to SFV RNAs. Presumably all encephaloviruses have a similar mechanism of viral RNA replication.

Electron microscopic studies of cells infected with SFV, Sindbis virus, Chikungunya virus, Venezuelan Equine Encephalitis virus and Western Equine Encephalomyelitis virus (see review, Mussgay, 1964) revealed 2 major morphogenetic steps in the formation of virus particles: (i) the appearance of nucleocapsids, and (ii) the acquisition (by budding) of an envelope at cellular membranes to form virus particles. Studies with ts mutants of Sindbis virus and SFV confirmed and expanded the above observation. Viral RNA presumably acts as a messenger for the synthesis of nucleocapsid and envelope or membrane proteins. It is not known which of these 2 proteins is synthesised first but the formation of nucleocapsids can occur in the absence of membrane protein formation, and vice versa.

Genetic studies with SFV have been disappointing, especially so since complementation occurred very efficiently between certain ts mutants of Sindbis virus but inefficiently with some mutants of SFV. Mutants of both viruses have similar physiological defects and were expected to show comparable genetic interactions. Recombination was not demonstrated between ts mutants

of Sindbis virus or SFV. Sambrook (1965) claimed to have obtained recombination between certain pairs of SFV ts mutants but this has not been confirmed.

It appears that ts mutants of SFV are somewhat refractory to genetic analysis. However, SFV (like other encephaloviruses) possesses an advantage over other riboviruses in that it can grow in a large number of cell types. Thus it is possible that the other valuable class of conditional lethal mutants, amber and ochre mutants (which are host-dependent but have not been reported for animal viruses), may be obtainable for SFV by the use of different cell types. Such mutants would be invaluable for the study of SFV genetics.

CONCLUSIONS

The single-stranded RNA of encephaloviruses (Fenner and Sambrook, 1964) has a molecular weight of about 2 million daltons and can code for about 10 proteins. This is calculated on the assumptions: (a) the genetic code is triplet and non-overlapping, (b) all or most of the viral genome codes for proteins, (c) such proteins contain on the average 200 amino-acids, and (d) single-stranded RNA of 2 million daltons contains about 6,000 nucleotides. In SFV infected cells, 4 proteins have been distinguished; one is RNA polymerase, one is found in the viral envelope and 2 in the nucleocapsid. Perhaps one more protein is required for the assembly of viral structural proteins into virions and another to inhibit host cell RNA synthesis. This accounts for only 6 proteins. Thus the replication scheme presented in figure 10-1 may be an over-simplification of the actual replication cycle; more than one enzyme may be involved in viral RNA synthesis and other functions essential for the growth of the virus have yet to be discovered.

It is clear that more mutants need to be isolated and more detailed studies made on selected mutants.

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